Applied Proteomics
Using the Peripheral Proteome to Identify a Surrogate Marker of Schizophrenia

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APPLIED PROTEOMICS: USING THE PERIPHERAL PROTEOME TO IDENTIFY A SURROGATE MARKER OF SCHIZOPHRENIA

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Para mis hijos *Guillermo y Jaime*.
A la memoria de mi abuelo *Abelardo*.

Abstract

Classical proteomic techniques have been used in medicine for biomarker discovery and have recently entered the arena of neurodegenerative disorders.

Biomarkers for disorders such as Huntington's, Parkinson's and Alzheimer's diseases are currently being developed and tested for use in early detection, disease progression, prognosis and response to treatment. However, psychiatric disorders have been less researched to date.

In this thesis, a classic proteomic approach was used (1) to examine alterations in molecular pathways determined by a well known high-risk schizophrenia (SCZ) gene (DISC1); (2) to assert the effects of antipsychotic medication in the brain and plasma of F344 rats; and (3) to canvass the plasma of psychotic patients searching for biomarkers of the disease.

It was found that DISC1 modulated the expression of dihydropteridine reductase, a key enzyme for biogenic amine synthesis and that of peptidyl-prolyl isomerase A, a protein involved in apoptosis.

Antipsychotic treatment in rats exerted an effect on glucose and lipid metabolic pathways, mitochondrial function, immune system response, neuronal migration, differentiation and apoptosis.

Alterations in calcium signalling pathways were detected in the plasma of psychotic patients, indicated by a significant reduction in plasma levels of gelsolin and an increment of S100B.

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Abbreviations

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2DPAGE	Two-dimensional polyacrylamide gel electrophoresis
2DIGE	2-Dimensional in gel electrophoresis
5-HT	5-hydroxytryptamine (Serotonin)

A

AC	Affinity chromatography
AC	Adenylate cyclise
ACE	Angiotensin I-converting enzyme
AD	Alzheimer's disease
ATP	Adenyl triphosphate
ATP5A	ATP synthase subunit alpha, mitochondrial
ARI	Aripiprazole

B

BAX	Bcl-2-associated X protein
BBB	Blood-brain barrier
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin

C

CHA2	Carbonic anhydrase
cAMP	Cyclic adenosine monophosphate
CAR-2	Carbonic anhydrase II
CAR/RXR-α	Androstane receptor (CAR, NR1I3)/retinoid X receptor alpha
CBF	Cerebral blood flow
CE	Capillary electrophoresis
CHGA	Chromogranin A (parathyroid secretory protein 1)
CHAPS	3 - ((3-cholamidopropyl) dimethylammonium)-1-propanesulfonic
CIT	Citalopram
CKK	Cholecystokinin
CLO	Clozapine
CMRglc	Cerebral metabolic rate for glucose
CMRO2	Cerebral oxygen metabolism
CNS	Central nervous system
COMT	Catechol-O-methyl-transferase
CRMP2	Collapsing response mediator protein 2
CSF	Cerebrospinal fluid
c-Src	Proto-oncogen tyrosine-protein kinase Src
CXADR	Coxsachie virus and adenovirus receptor

D

DA	Dopamine
DHPR	Dihydropteridine reductase
DISC 1	Disrupted-in-schizophrenia-1
DIGE	Two-Dimensional differential gel electrophoresis
DJ-1	Parkinson disease autosomal recessive, early onset 7 (PARK7)
DRD1	Dopamine receptor D1
DRD2	Dopamine receptor D2
DSM-IV	Diagnostic and statistical manual of mental disorders, 4th edition

DTNBP1	Dysbindin
DTI	Diffusion tensor imaging
DTT	Dithiothreitol

E

ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked immunosorbent assay
ENO2	Neuron-specific enolase
ERK ½	Extracellular signal-regulated kinases 1/2
ESI	Electrospray ionisation
ESR1	Estrogen receptor-1

F

FDR	False discovery rate
fMRI	Functional magnetic resonance imaging

G

G3P2	Glyceraldehyde-3-phosphate dehydrogenase
GABA	Gamma-aminobutyric acid
GAF	Global assessment of function
GLU	GLU
GRM3	Metabotropic glutamate receptor 3
GSK3	Glycogen synthase kinase 3

H

HAL	Haloperidol
HD	Huntington's disease
HPLC	High–performance (or pressure) liquid chromatography
HCRT	Orexin A (Hypocretin-1)
HSV-2	Herpes simplex virus type 2

I

iCAT	Isotope-coded affinity tag
ICD-10	International classification of diseases, 10th revision
IDH3B	Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial
IDO	Indoleamine 2,3-dioxigenase
IEF	Isoelectric focusing
IEX	Ion exchange chromatography
IFN	Interferon
IL	Interleukin
IQ	Intelligence quotient
iTRAQ	Isobaric tags for relative and absolute quantification
IPG	Immobilized pH gradient

J

JAK/STAT	Janus kinase/signal transducers and activators of transcription

K

KCC2	Neuron-specific K-Cl cotransporter
KCRB	Creatine kinase B-type
KYNA	Kynurenic acid

L

LC	Liquid chromatography
LC/MS/MS	Liquid chromatography tandem mass spectrometry
LPS	Lipopolysaccharide
LTD	Long term depression
LTP	Long term potentiation

M

MALDI	Matrix-assisted laser desorption/ionisation
MHC	Major histocompatibility complex
MKK7	MAP2K7, mitogen activated protein kinase kinase 7
MPTP	Mitochondrial permeability transition pore
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRS	Magnetic resonance spectroscopy
MS	Mass spectrometry
MS/MS	Mass selection/mass separation
MudPIT	Multi-dimensional liquid chromatography coupled to tandem mass spectrometry
MWt	Molecular weight

N

NAA	N-acetylaspartate
NCAM1	Neural cell adhesion molecule 1
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NMR	Nuclear magnetic resonance
NOS	Nitric oxide synthase
NRG1	Neuregulin 1
NRGN	Neurogranin
NT	Neurotransmitter
NTS	Neurotensin

O

OD	Optical density
OLA	Olanzapine
OR	Odds ratio
OX	Orexin/hcrt-1 hypocretin receptors

P

PBMC	Peripheral blood mononuclear cells
PCP	Phencyclidine
PD	Parkinson's disease
PDA	Piperazine diacrylamide
PET	Positron emission tomography
PGAM1	Phosphoglycerate mutase 1
pI	Isoelectric point
PKA	Protein kinase A
PKM2	Pyruvate kinase isoenzymes M1/M2
PNRC2	Proline-rich nuclear receptor coactivator 2
PNS	Peripheral nervous system
PP	Protein phosphatase

PPIA	Cyclophilin A
PZP	Pregnancy zone protein or alpha-1-macroglobulin

R

RAR	Retinoic acid receptor
RBC	Red blood cells
RIS	Risperidone
ROC	Receiver operating characteristic
ROI	Region of interest
RPC	Reversed phase chromatography
RSK	Ribosomal S6 kinase
S6K1	Ribosomal protein S6-p70-protein kinase

S

S100B	S100 calcium binding protein B
SCAN	Schedules for clinical assessment in neuropsychiatry
SCZ	Schizophrenia
SDHA	Succinate dehydrogenase subunit A
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELDI-TOF-MS	Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry
SEM	Standard error of mean
SPECT	Single photon emission computed tomography
SPSS	Statistical package for the social sciences

T

TBS	Tris buffered saline
TBS-M	Tris buffered saline-milk
TBS-T	Tris buffered saline-tween
TCF4	Transcription factor 4
TCR	T cell (lymphocyte) receptor
TDO	Tryptophan 2,3-dioxygenase
TGF-β	Transforming growth factor beta
TH	T-helper
TIFF	Tagged Image file format
TIP1	Triosephosphate isomerase
TEMED	Tetramethylethylenediamine
TMT	Tandem mass tags
TNF	Tumor necrosis factor
TRH	Thyrotropin-releasing hormone
TRP	Tryptophan
Trk	Tropomyosin-receptor-kinase
TTR	Transthyretin

U

UCHL1	Ubiquitin carboxyl-terminal esterase L1

V

VDAC 1/2	Voltage-dependent anion-selective channel protein 1/2

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Life enduring a severe mental illness and the side effects of its treatment is hard enough, but volunteering to give a sample of tissue in the hope that other people's suffering will eventually end or be reduced is a sacrifice that not many of us could afford to do in similar circumstances. I have no words left to acknowledge the tremendous contribution patients have made, and will continue to make, to psychiatric research.

And so, to them, to their families, carers and friends: Thank you.

CHAPTER 1: Introduction

1.1. Schizophrenia

1.1.1. Definition

In 1911 Bleuler coined the word 'Schizophrenia' (fragmented mind) (SCZ) to describe a condition that afflicted a group of psychiatric asylum patients who presented with 'ambivalence', 'autism', 'affective incongruity' and 'disturbance of association of thought'. He had followed on Morel's 1860 'démence précoce' and Kraepelin's 1896 'dementia praecox' despairing prognostic concepts, and his description of that clinical entity was used by Schneider in 1959 to establish what was termed the 'first rank' symptoms: auditory hallucinations, delusional perception, delusions of passivity (thought insertion, withdrawal and broadcasting; made feelings, impulses and actions) and somatic passivity.

Symptoms present in psychotic disorders can be clustered into five main categories: (i) positive-symptom dimension (delusions and hallucinations); (ii) negative-symptom dimension (lack of motivation, reduction in spontaneous speech, and social withdrawal); (iii) cognitive-symptom dimension (difficulties in memory, attention, and executive functioning); (iv) depressive symptoms and (v) manic symptoms. The term schizophrenia is applied to a syndrome characterised by long duration of symptoms, bizarre delusions, negative symptoms, and few affective symptoms (non-affective psychosis) (van and Kapur, 2009).

Nowadays, SCZ is conceptualised among clinicians as a polygenic and multifactorial serious mental disorder that tends to manifest itself in early adulthood, impairing the intellectual, emotional and social development and function of the individual.

1.1.2. Diagnosis

The diagnosis of SCZ remains based on clinical judgement. Symptoms of SCZ are traditionally described and categorised as either being part of newly emerging psychopathology, the so called 'positive' symptoms (i.e. thought disorder, hallucinations and delusions); or resulting in the sequestering of intellectual and emotional functions by the disorder itself and hence called 'negative' symptoms (i.e. apathy, social withdrawal, self-neglect, loss of motivation, emotional blunting and poverty of speech); or as being responsible for altering normal cognitive functioning (i.e. episodic memory loss) (Picchioni and Murray, 2007). The study of cognitive symptoms is becoming increasingly important in SCZ research and the appropriateness of including them as diagnostic criteria has been put forward recently (Keefe, 2007).

This state of abnormal salience and reality distortion (positive symptoms), social and emotional deficiencies (negative symptoms) and cognitive disturbances, results in observable

changes in behaviour which are deemed to be maladaptive and cause great distress to sufferers and carers.

Whereas elsewhere in medicine diagnoses are being revised in the light of biological markers of disease process as they are being discovered, the diagnosis of SCZ remains empirical and currently relies solely on clinical assessment, symptom stability and pattern recognition by highly trained and skilled clinicians. With the introduction of semi-structured interviewing techniques and the use of operationalised diagnostic criteria, the process of diagnosis has become standardised and highly reliable once psychotic symptoms are elicited and firmly established.

1.1.3. Epidemiology

The lifetime risk of developing SCZ is estimated to be on average 0.7% (Saha *et al.* 2005), while the incidence of SCZ is thought to range from 7-40/100,000 (new) cases per year depending on whether broad or narrow diagnostic criteria are applied. A meta-analysis of 55 studies from 33 different countries found a median annual incidence rate of 15.2/100,000 and identified urbanicity, migration and male gender as the factors contributing the most to incidence variation (McGrath et al. 2004). SCZ is a worldwide prevalent disorder with areas of higher and lower prevalence and a median point prevalence of 4.6/1000 (CI 1.9-10/1000), a median period (up to a year) prevalence of 3.3/1000 (CI 1.3-8.2/1000) and a median lifetime prevalence of 4/1000 (CI 1.6-12.1/1000) (Saha *et al.* 2005).

Persons with SCZ used to be thought to 'drift' to inner city areas where less financial resources to sustain oneself are needed and where a heightened population density grants a certain degree of anonymity, making the psychotic patient less conspicuous and preventing involuntary hospital admission or detention. Nonetheless, it is possible that through some unknown factor such as illicit substance use, pre and perinatal health, social stress and adversity, environmental toxins, infectious diseases, nutritional variation or a combination of all of them, urbanicity may actually cause psychotic symptoms to appear (Pedersen and Mortensen, 2001).

Migration has also been repeatedly purported to increase the risk of developing SCZ, particularly when the direction of migration is from a black majority country to a white majority one, or to areas with lower density of people with the same ethnic background as the migrant, providing additional support for the role of social factors in the aetiology of SCZ (Cantor-Graae, 2007).

1.1.4. Environmental risk factors

The role played by environmental factors in the onset and perpetuation of psychotic disorders has been clearly established. It is widely accepted that environmental agents can modulate gene function either by epigenetic mechanisms (i.e. by inducing methylation of specific gene sites and thus activating or inhibiting its expression), by driving specific changes in splicing or by protein modification (i.e. post-translational modification).

These external factors wield their influence at different time points of brain development. In the antenatal stage, maternal infections, severe adverse life events and nutritional deficiencies in early pregnancy appear to be associated with higher risk of SCZ in the offspring (Penner and Brown, 2007) which may suggest a dysfunction on the maternal immune and/or stress responses. Older paternal age at conception is also thought to increase the risk of SCZ (Malaspina, 2001). During the perinatal period, obstetric complications resulting most likely in a relatively prolonged period of hypoxia have shown correlation with subsequent risk of developing SCZ (Geddes and Lawrie, 1995).

There is also a season of birth effect by which patients with SCZ are more likely to have been born in late winter or early spring and which increases with latitude and severity of the winter season (Torrey and Miller, 1997). During childhood, parental separation or death (Morgan et al. 2007), childhood trauma (Read et al. 2005), adverse child rearing (Tienari et al. 2004), urbanicity, migration and infection are thought to contribute to raising the risk for SCZ. In adolescence, social adversity, stressful life events (Norman and Malla, 1993), and the early and heavy use of cannabis (Semple et al. 2005) have been associated with increased the risk of the disorder. The manner in which these factors interact with each other and with a given underlying genetic predisposition is largely unknown and so are the neurobiological pathways involved.

1.1.5. Genetics

A genetic basis for SCZ has long been suspected (Kendler and Diehl, 1993). SCZ is widely considered to be a polygenic and highly heritable disorder with 83% of the variance in liability due to additive genetic factors and the remaining 17% to unique environmental factors (Cannon et al. 1998), and has been described as a 'threshold character whose phenotypic appearance would depend on the both the number of genes present and the amount of stress' (Gottesman and Shields, 1967).

Early institutional records established in the past century during the transition from the asylum system to the current more proactive approach to treating mental disorders,

recorded this familial segregation of mental illnesses. Later on, adoption studies reliably illustrated that the risk of suffering SCZ was increased by being the offspring of affected biological parents (Heston, 1966; Kety, 1983). Twin studies consistently found a higher concordance rate between monozygotic (40-50%) than between dizygotic twins (10-15%) (Sullivan *et al.* 2003).

Chromosomal abnormalities in affected individuals and their families have consistently been described and replicated as conferring a higher risk of suffering SCZ (MacIntyre et al. 2003). Examples of this are the deletion of 22q11 occurring in the Velo-Cardio-Facial Syndrome (VCFS) which contains the genes catechol-O-methyltransferase (COMT) and T-box 1 (TBX1); the balanced reciprocal translocation of 1q42/11q14 affecting the disrupted-in-schizophrenia-1 (DISC1) gene; and alterations in the X chromosome.

Genetic research advanced greatly after the complete sequencing of the entire human genome 10 years ago (Lander et al. 2001; Venter et al. 2001) and the conclusion of numerous linkage analysis studies, in which information from families with multiple affected individuals were used.

As a result of these efforts, several regions of the genome thought to be associated with conferring risk for developing SCZ were identified. These are, in descending order of importance: 2p12-q22, 5q23-q34, 3p25-p22, 11q22-q24, 6pter-p22, 2q22-q23, 1p13-q23, 22pter-q12, 8p22-p21, 6p22-p21, 20p20-p11, 14pter-q13, 16p13-q12, 18q22-qter, 10pter-p14, 1q23-q31, 15q21-q26, 6q15-q23 and 17q21-q24 (Lewis et al. 2003). Regions 10p15-p13, 2 centromere and 22q12 were later identified in a genome wide scan in sibling pairs (DeLisi et al. 2002). Linkage analyses are of limited value. They signal over 4000 potential candidate genes thought to be involved in the disease process, are unable to specifically identify the individual risk genes and lack statistical power to detect small effect size genes.

Association studies, which are usually done in samples of unrelated individuals, were thus conducted to try to identify which specific genes contributed to the disorder. They complemented linkage studies and are theoretically capable of identifying smaller effect size genes. Some of the genes that have been frequently detected are: neuregulin 1 (NRG1), dysbindin (DTNBP1), dopamine receptors 1-4 (DRD1-4), DISC1, catechol-O-methyl-transferase (COMT) and metabotropic glutamate receptor 3 (GRM3) (Tandon et al. 2008). In a recent genome wide association study published in Nature, Steffanson and colleagues found a significant association with markers in the major histocompatibility complex (MHC) region on chromosome 6p21.3-22.1, a marker located upstream of the neurogranin (NRGN) gene on 11q24.2 and a marker in intron 4 of transcription factor 4 (TCF4) on 18q21.2 (Stefansson *et al.*

2009). The MHC region is crucial to the normal functioning of the immune response, whereas NRGN and TCF4 are thought involved in brain development, memory and cognition.

Association studies are limited by the number of false positive and false negative results, the difficulty in replicating exactly the same markers and haplotypes in different clinical samples, and by population stratification. Table 1 provides a summary of the main findings in the genetics of SCZ. Thus far, no single gene has been unequivocally being linked with the disorder as being either sufficient or necessary.

Moreover, the molecular pathways and cellular functions emerging from genetic studies thought to be altered in SCZ are still largely unknown.

1.1.6. Gene-Environment interaction (G x E)

The high heritability (around 80%) of schizophrenia is not only due to genetic influences but also genetic epidemiological studies have shown that gene–environment interaction in schizophrenia is common (gene–environment interaction).

Efforts are being made in bringing together the different disciplines needed to examine models of disease causation based on various aspects of gene–environment interplay. Epigenetic factors susceptible to environmental influence might also affect twin heritability estimates. Meta-analytic work has suggested that paternal age above 40 years is associated with schizophrenia, indicating that epigenetic mechanisms might have a role (van and Kapur, 2009).

Co-participation of environmental exposure and background genetic vulnerability is thought to be common in multifactorial disorders such as psychosis. A well-known example of this is alcohol sensitivity in Oriental people which is strongly regulated by genetic polymorphism of the aldehyde dehydrogenase (ALDH2) gene.

Recent G x E findings in psychiatry suggest that genes are likely to influence disorder mostly indirectly (impacting on physiological pathways and modifying the likelihood of developing a psychiatric disorder), rather than as direct causes of disorder per se.

For example, the gene 5-HTTLPR has been shown to interact with environmental adversity to cause depression, reflecting underlying developmental mechanisms that affect the structural connectivity and functional interactions of a neural circuit involved in the regulation of emotional reactivity and extinction of fear. In schizophrenia, research has shown that the valine allele carriers of a functional polymorphism in the catechol-*O*-methyltransferase gene (COMT Val^{158}Met), an important enzyme regulating prefrontal dopamine turnover, predicted

increased dopamine synthesis in the midbrain, suggesting that this allele may increase the risk for schizophrenia in interaction with stress and dopamine agonist drugs. Several studies suggest that valine-allele carriers may indeed be more sensitive to the psychotogenic effects of drugs of abuse or stress.

The most solid evidence for an association with schizophrenia and related psychosis outcomes is for paternal age, migration, urbanicity, and cannabis use, the latter two particularly in the case of exposure during development. The molecular mechanisms underlying this G x E interaction are not clear but it has been suggested that the synergistic effects result in a "sensitization"of mesolimbic dopamine neurotransmission. It is known that the genetic risk for schizophrenia is associated with underlying alterations in the dopamine system like increased dopamine synaptic availability, increased striatal dopamine synthesis, and increased dopamine reactivity to stress. Stress and dopamine agonist drugs release dopamine in mesolimbic areas and facilitate subsequent sensitization of dopaminergic pathways. Finally, there is evidence from human and animal studies that developmental trauma, defeat stress associated with ethnic minority group, prenatal hypoxia, and prenatal maternal immune activation have lasting effects on dopamine neurotransmission (van *et al.* 2008).

Table 1. Summary of replicated genetic findings in SCZ.

	Family studies	Linkage areas*	GWAS in siblings	GWAS
Identified gene regions	22q12	2p12-q22	10p15-p13	6p21.3-22.1 (MHC)
	1q42/11q14	5q23-q34	2 centromere	11q24.2 (NRGN)
		3p25-p22	22q12	18q21.2 (TCF4)
		11q22-q24		6p22.3 (DTNBP1)
		6pter-p22		5q35.1 (DRD1)
		2q22-q23		11q23 (DRD2)
		1p13-q23		3q13.3 (DRD3)
		22pter-q12		11p15.5(DRD4)
		8p22-p21		22q12 (VCFS)
		6p22-p21		22q11.21-q11.23(COMT)
		20p20-p11		7q21.1-q21.2 (GRM3)
		14pter-q13		
		16p13-q12		
		18q22-qter		
		10pter-p14		
		1q23-q31		
		15q21-q26		
		6q15-q23		
		17q21-q24		

*Linkage areas are listed in descending order of importance.

1.1.7. Neurochemistry

a. Dopamine

This catecholamine synthesized in 1910 by Barger and Ewens at the Wellcome laboratories in London and recognised as a key NT by Arvid Carlsson in 1958 has been thought to be involved in the pathological process of SCZ for the most part of the past 50 years.

The main supporting evidence of its causal link to psychotic symptoms is the relatively effective dopaminergic blockade exerted by antipsychotic medication, and the excess dopamine release observed in the striatum and its associated acute psychotic state following amphetamine intake (Laruelle et al. 1996).

A theoretical model for SCZ based on hypoactive dopaminergic activity in mesocortical regions and hyperactive dopaminergic activity in mesolimbic areas has been proposed to explain the emergence of negative/cognitive symptoms and positive symptoms of the disorder respectively (Davis et al. 1991). Alternative (or complementary) models invoke hypoactive tonic basal activity with hyperactive phasic dopaminergic responses to external stimuli, and differences in lateralization of neuronal transmission (Keshavan *et al.* 2008).

Prefrontal, striatum and temporal lobe dopaminergic hypofunction mediated by genetic variation in dopamine-dependent genes results in deficits in Intelligence quotient (IQ), attention, executive function and working memory (Tan et al. 2009). These alterations can also be found in relatives of affected individuals with higher prevalence than in the general population.

In contrast, a dopaminergic hyperfunction in the striatum (caudate and putamen), in terms of increased dopamine release and striatal dopamine receptor density may be responsible for deficits seen in procedural learning of fine motor actions, spatial working memory, attentional processes, increasing premature and perseverative responding (Simpson et al. 2010). This cognitive impairment appears early during the course of the disorder and is pervasive in nature, becoming most debilitating in the late stages of the disorder by affecting adequate social functioning.

Additional evidence of association between cognitive functions and dopamine is that a small improvement in cognitive symptoms was detected after 2 months of antipsychotic treatment during the CATIE trial (Keefe *et al.* 2007).

b. Glutamate (GLU)

Glutamic acid is a non-essential aminoacid which is ubiquitously present in the nervous system of vertebrates acting as an excitatory NT via N-methyl-D-aspartate (NMDA) receptors. Glutamatergic dysfunction is also thought to play an important role in the pathogenesis of the disorder. NMDA antagonists, such as ketamine and phencyclidine (PCP or 'angel dust') are capable of triggering psychotic symptoms and reduced expression of glutamate receptors has been observed in the pre-frontal cortex and hippocampus of patients with SCZ in post-mortem studies. Low concentrations of glutamate in the cerebrospinal fluid (CSF) of patients with SCZ (Kim *et al.* 1980), support the theory that hypofunction of the glutamatergic cortico-estriatal pathway may lead to the emergence of psychotic symptomatology by altering the filtering of sensory information at the thalamus. In addition, since glutamate is also involved in long-term potentiation, its purported effect is mainly thought to be associated with cognitive deficits and negative symptoms (Keshavan *et al.* 2008).

c. Gamma-aminobutyric acid (GABA)

A product of glutamate in GABA-ergic neurons, this inhibitory NT binds to receptors that are either part of a ligand-gated ion channel complex ($GABA_A$) or G protein-coupled metabotropic receptors ($GABA_B$). $GABA_A$ causes the opening of chloride ions (CL^-) channels altering the membrane potential and depolarizing (excitatory action) the neuron during the neonatal stage by allowing chloride out of the cell and then *switching* the flow during normal development in the adult brain, allowing chloride to enter the neuron and thus, hyperpolarizing it (inhibitory action). Post-mortem studies have shown reduced levels of GABA expression in prefrontal cortex and a possible compensatory upregulation of $GABA_A$ receptors. These alterations seem to correlate with working memory impairment in SCZ (Keshavan *et al.* 2008).

d. Serotonin (5-HT)

The serotonergic hypothesis of SCZ was first suggested in 1954 (Wooley and Shaw, 1954) based on the 5-HT antagonism effect that LSD (D-lysergic acid) had in the CNS. Although the initial hypothesis was of serotonin deficiency, it was soon reframed as of excessive serotonergic transmission. Hallucinogenic drugs are all partial 5-HT_2 agonists and recent evidence shows that activation of 5-HT_{2A} receptors in cerebral cortex increases glutamatergic transmission by enhancing glutamate release in neocortex (Aghajanian and Marek, 1997).

e. Other

Cholinergic, muscarinic, noradrenergic and endocannabinoid activity has also been shown to be altered in post-mortem studies of brain tissue of people with SCZ but the evidence to date, direct or indirect, is less compelling and rather incomplete.

1.1.8. Neuropathology

Post-mortem studies have their own limitations in psychiatric research (Lewis, 2002). Unlike neurodegenerative disorders like Parkinson's or Alzheimer's diseases, SCZ lacks pathognomonic lesions in the brain of affected individuals.

Establishing prospective, antemortem assessments to affected and control groups both is difficult. Clinical diagnosis, psychopathology measurements at time of death, the effect of age heterogeneity, chronicity of the disorder, use of medication and/or illicit drugs, the impact of past or recent overdoses, manner and cause of death, suicide-related factors and agony time amongst others need to be reliably documented. In addition, post-mortem variability induced by post-mortem interval, preservation technique and freeze-thaw cycles for instance, are likely to induce pH changes that may affect mRNA transcripts by degrading them. This degradation may result in an abnormal structural or functional protein expression that might not be directly related to the disorder itself.

Details of neuropathological changes in SCZ, whether pathognomonic or not, remain elusive. For well over 100 years, researchers have attempted to identify and describe changes in the brain associated with the disease and other neuropsychiatric disorders such as Alzheimer's disease and Parkinson's disease have been already mapped to specific brain changes. More recently, there appears to be emerging evidence of a specific neuropathology associated with schizophrenia but the details and significance of these changes is not yet well understood.

a. Gross pathology

Post-mortem studies of the brain in people with SCZ have shown lower overall weight; alterations in brain symmetry; enlarged ventricles and structural changes in hippocampus, frontal and temporal lobe regions; thinning of grey matter in absence of gliosis; abnormal neuronal migration; dysfunctional apoptosis and synaptic and dendritic alterations (Brown *et al.* 1986; Harrison, 1999; Iritani, 2007; Selemon *et al.* 2002; Selemon and Goldman-Rakic, 1999). Enlarged lateral and third ventricles, decreased brain size, cortical volume (especially in

the temporal lobes) and hippocampal volume have also been reported in first episode patients (Steen *et al.* 2006).

b. Neurodegenerative neuropathology

Signs of neurodegeneration in SCZ have not yet been conclusively found. There are no discrete lesions such as neurofibrillary tangles or plaques despite some patients experiencing profound cognitive deficits. Evidence of gliosis has been reported (Stevens, 1982) but also rebutted elsewhere (Arnold *et al.* 1996; Schmitt *et al.* 2009), making the argument that a general inflammatory process occurs and accounts for the emergence symptoms unlikely. The generally accepted view is that gliosis is a coincidental or over imposed feature, probably resulting from focal lesions and infarcts in a subset of patients. Nonetheless, absence of gliosis does not preclude aberrant plasticity or neurotoxic changes in SCZ.

c. Cellular changes

Neurons in hippocampus (particularly those in glutamatergic pathways) and in the prefrontal cortex (GABAergic neurons mainly) are smaller than in the general population, suggesting smaller axons and dendrites resulting in fewer and less active synaptic connections. These changes are supported by microarray studies showing changes in presynaptic and synaptic functions (Mirnics *et al.* 2000; Mirnics *et al.* 2001).

SCZ has also been associated with volume and neuronal changes in the mediodorsal nucleus and the pulvinar in the thalamus in postmortem studies (Byne *et al.* 2002). The former has extensive reciprocal connections with the prefrontal cortex and it may be that one of those areas is the primary focus of the common changes observed between them.

These changes are observed in first episode patients and hence are thought to be independent of medication, albeit recent evidence from animal studies suggest that typical antipsychotic treatment may also have deleterious effects on neurotransmitter pathways and cognition (Terry, Jr. *et al.* 2005; Terry, Jr. *et al.* 2007b; Terry, Jr. *et al.* 2007a; Vernon *et al.* 2011).

d. Neural connectivity

Neuronal and synaptic changes identified mainly in the dorsolateral prefrontal cortex and in the hippocampus appear to indicate that SCZ is a disorder of neural connectivity. In the hippocampus, synaptic and dendritic alterations are suggested by the decreased expression of

SNAP-25 (synaptophysin) and others pre-synaptic proteins, which reflects a lowered synaptic density (Harrison and Eastwood, 2001). In the dorso-lateral prefrontal cortex, levels of SNAP-25 have been reported to be reduced in SCZ in many studies (Davidsson *et al.* 1999; Glantz and Lewis, 1997; Honer *et al.* 1999; Karson *et al.* 1999; Perrone-Bizzozero *et al.* 1996). Based on this and other synapse-related proteins reported reductions, the 'reduced neuropil hypothesis' of SCZ (Selemon and Goldman-Rakic, 1999) purports that axon terminals, dendritic spines and shafts of most cortical synapses are reduced in SCZ, without any apparent loss of neurons.

1.1.9. Neuroimaging

Neuroimaging has been fundamental in determining biological correlates of SCZ. Magnetic resonance imaging (MRI) uses a strong magnetic field to excite the hydrogen atoms contained in the extra and intracellular fluids and detects the subsequent alignment resulting from the magnetization, transforming the signal into a visual representation of organs, soft tissues and bone structure. A series of structural findings have appeared to be consistent in SCZ; amongst them, an overall reduction in brain volume, thinning of grey matter, ventricle enlargement, and smaller temporal lobe structures like hippocampus (Heckers, 2001), amygdala and superior temporal gyri; smaller prefrontal cortex, thalamus, anterior cingulated and corpus callosum (Keshavan *et al.* 2008).

Patients with SCZ also show abnormal cerebral symmetry (Crow et al. 1989), which is inversely correlated to early age of onset; and hemispheral dominance. Some of these changes appear in the early, prodromal stages of the disorder and are stable throughout the course of the illness although, in some cases, there seem to be a neurodegenerative component to the progression of these anatomical alterations. These changes are also seen to a lesser extent in other psychotic disorders such as bipolar affective disorder (BPAD) (Stoll et al. 2000) and in unaffected relatives, all of which prevent them from being considered pathognomonic or diagnostically relevant. Moreover, although most of these changes are present at illness onset, antipsychotic medication has been shown to induce structural changes in basal ganglia by increasing its volume (typical antipsychotics) (Navari and Dazzan, 2009) or decreasing it (atypical antipsychotics) while increasing thalamus and cortical grey matter volumes (Scherk and Falkai, 2006), suggesting a region-specific modulatory effect of antipsychotic treatment which could be mediated by their action on altered underlying molecular pathways.

Positron emission tomography (PET) and single photon emission tomography (SPECT) is used in the study of a range of physiological processes in vivo like cerebral blood flow (CBF),

cerebral metabolic rate for glucose (CMRglc), cerebral oxygen metabolism ($CMRO_2$), pre- and postsynaptic receptor density, NT release, enzyme activity and protein synthesis amongst others, by using positron emitter tracers such as carbon-11 (^{11}C, $t_{1/2}$ = 20 min), nitrogen-13 (^{13}N, $t_{1/2}$ = 10 min), oxygen-15 (^{15}O, $t_{1/2}$ = 2 min) and fluorine-18 (^{18}F, $t_{1/2}$ = 110 min) (a substitute of hydrogen). In a recent review of PET studies, schizophrenic patients were shown to have reduced glucose metabolism, reduced blood flow in the dorsolateral prefrontal cortex, and reduced capability to increase blood flow in response to frontal lobe cognitive testing (Giovacchini et al. 2011). Similarly, they also showed decreased flow in the fronto-thalamic-cerebellar circuitry as well as a greater reduction in binding of ^{11}C-raclopride and ^{123}I-iodobenzamide (DRD_2 antagonists) following amphetamine challenge, signalling a proportional increase of extracellular dopamine in schizophrenic subjects, which in turn correlates with the emergence of positive symptomatology.

A most recent technique, diffusion tensor imaging (DTI) uses the fact that fluids move more freely and rapidly through the longer axis of the structure that contains them to create in vivo images of internal structures. The resolution of the technique is such that axon fibres can be individually identified (tractography). Recently applied to neuroscience, this technique has shown reduced structural integrity in white matter tracts in the corpus callosum, the cingulum, arcuate fasciculus and uncinate fasciculus in patients with SCZ (Kubicki et al. 2007).

1.1.10. Infections and immunology

Although the evidence for the involvement of infectious agents and immune system dysregulation in SCZ is at times contradictory due to confounding factors such as chronicity and severity of the symptoms, use of antipsychotic medication, smoking and poor general physical health in chronic patients, there is some evidence suggesting a link between infections and the emergence of psychotic phenomena.

Early ecological studies in influenza epidemics and subsequent birth cohort studies have shown an association between gestational age and risk of SCZ which appears to be higher if the infection occurred during the first half of the gestation (Brown and Derkits, 2010). Infections mediated by toxoplasma gondii and the herpes simplex virus type 2 (HSV-2) are also considered to increase the risk of SCZ with an estimated odds ratio (OR) of 2.6 and 5.0 respectively, contributing to 13% and 6% of the attributable proportion (AP) (the proportion of cases who would not have developed the disease if a cause were removed) of the cases (influenza virus has an OR of 3 and an AP of 14% of the cases). The issue of whether unique effects mediated by the responsible pathogen or a common mechanism lies at the heart of the

molecular alterations that eventually cascade into psychotic symptomatology is still unresolved. Infections associated with SCZ, as the ones mentioned above, differ form each other in terms of incubation time, duration and severity of symptoms, immune response elicited, capacity to cross placenta and blood-brain barrier and impact on brain development (Brown and Derkits, 2010), all of which seem to support the notion that individual infections and their associated unique characteristics are responsible for the heightened risk, however this is far from accepted by the majority of researchers.

The alternative explanation is that infections would act through common pathways to alter brain development and increase susceptibility to SCZ involving mainly the host response to infection. This response can be either cell-mediated immunity (Type-1) directed against the intracellular pathogens, or through production of antibodies against extracellular pathogens, the so-called humoral (Type-2) response.

Cytokines are secreted by immune cells (including glia in the CNS) as part of Type-1 and Type-2 responses and act as markers of infection and inflammatory states and are soluble peptides that regulate all types and all cellular components of the immune system. They are also involved in critical roles during normal brain development. Cytokines regulate the expression of MHC Class 1 proteins in the cellular membrane, which main function is to present the antigen to the T lymphocytes for the subsequent antibody production and to contribute to synaptic remodelling and plasticity.

Pro-inflammatory Type-1 activating cytokines such as Interleukin-2 (IL2), IL6, IL8, IL18, Interferon-γ (IFN-γ) and Tumour Necrosis Factor-α (TNF-α) stimulate cellular mediated immune response and are counterbalanced by anti-inflammatory Type-2 cytokines such as IL4, IL10 and IL13 which induce antibody responses. Patients with SCZ often show a blunted production of Type-1 cytokines and activation of Type-2 immune response in the early stages of the disorder (Muller and Schwarz, 2006). This is supported by pharmacological studies showing the therapeutic effects of cyclo-oxygenase-2 inhibitors (Riedel et al. 2005), and by the fact that neuroleptic treatment normalizes the production of Type-1 cytokines (Na and Kim, 2007). The therapeutic effect of COX-2 inhibitors seems to disappear in the later stages of the disorder (Muller et al. 2010). It appears that in the short-term, SCZ is particularly associated with disruptions in gene transcription, metal ion binding, RNA processing and vesicle-mediated transport, while the long-term illness is associated with inflammation, stimulus-response and immune function alterations (Narayan et al. 2008).

Raised cytokines levels secondary to maternal infections have been associated not only with developmental brain damage in the offspring (Dammann and Leviton, 1997) but also with chorioamnionitis, periventricular leukomalacia and cerebral palsy, and are thought to be

responsible for microglia and astrocyte activation resulting in increased levels of nitric oxide (NO) and excitatory aminoacids, disruption of maturation of oligodendrocytes and subsequent appearance of white matter abnormalities in patients with SCZ (Davis et al. 2003).

Raised levels of the cytokines IL1β, IL6, IL8 and TNF-α occur with most infections and have been associated with psychotic disorders (Brown and Derkits, 2010). One of the most robust findings in the immunological studies of SCZ is the shift from T-helper 1 (Th1)-like cellular to Th2-like humoral immune response (Monji *et al.* 2009). IL6 is discharged into the bloodstream after muscle contraction and acts to increase the breakdown of fats and to improve insulin resistance.

This Type-1/Type-2 imbalance is played out in the CNS by microglial cells and astrocytes, since the former are derived from peripheral macrophages and secrete mainly Type-1 cytokines while the latter secrete Type-2 such as IL10. To support this claim, a marker of astrocyte activation which is thought to be associated with serotonin levels in brain and considered as one of the most potent trophic factors for serotonergic neurons, S100B, has been consistently found increased in the early stages of SCZ and is associated with structural damage as part of a neurodegenerative component of the disorder (Zhang *et al.* 2010b). In addition, astrocytes are the main source of the only known naturally occurring N-Methyl-D-Aspartate (NMDA) receptor antagonist in the human CNS, kynurenic acid (KYNA) (Schwarcz and Pellicciari, 2002); a product of Tryptophan (TRP) metabolism, which is metabolized by the enzymes Indoleamine 2, 3-dioxigenase (IDO), and is induced by Type-1 cytokines and by tryptophan 2 3-dioxygenase (TDO), and has been found overexpressed in the brain of schizophrenic patients (Miller et al. 2004). KYNA accumulates following astrocyte activation and may lead to the emergence of psychotic phenomena through impaired glutamatergic transmission (Muller and Schwarz, 2006).

NMDA antagonists such as phencyclidine (PCP) and ketamine are known to induced microglial activation in rats (Nakki et al. 1995). Microglial dysfunction as a result of glutamate-mediated toxicity has been reported in a study of elderly schizophrenic subjects (Chang *et al.* 2007) and microglial activation has also been found in post-mortem samples of frontal cortex and hippocampus of patients suffering from SCZ (Bayer et al. 1999). Despite these arguments in favour of a primary glial activation process, alternative explanations have also been offered for the changes observed and thus, when the authors of a post-mortem study on the brains of patients with SCZ and depression who committed suicide found an increased in microglial activation, they attributed it to a pre-suicide stress reaction (Steiner et al. 2008) and in one imaging study, the authors hypothesize that the microglial activation seen in the brains of patients might have been secondary to neuronal loss (van Berckel et al. 2008). Despite this

limitations and potential alternative explanations, a primary activation process cannot be totally excluded.

In preclinical studies, the alterations of brain development seen in maternal immune activation animal models are very similar to the ones observed in DISC1 and NRG-1 knock-down animal models and in a recent paper, Seshadri and colleagues demonstrated that DISC1, like NRG1, is expressed in neurons, astrocytes and microglia and exert their effects via a common pathway involving heterodimers of the receptors tyrosine-protein kinase ErbB2 and ErbB3 in a mechanism that appears to involve Akt signalling (Seshadri et al. 2010).

Impaired Akt signalling has already been associated in SCZ (Bajestan et al. 2006; Mathur et al. 2010; Norton et al. 2007; Thiselton et al. 2008) and with obstetric complications (Joo et al. 2009), while ErbB3 modulates antigen presenting cell function and Type 1 Diabetes risk (Wang et al. 2010), which in a recent study has been linked to SCZ (Copeland et al. 2010), echoing mounting evidence for conceptualizing SCZ as a systemic disorder (Dickinson and Harvey, 2009), particularly of insulin resistance and chronic inflammation.

1.1.11. Pharmacological treatment of schizophrenia

The serendipitous introduction of chlorpromazine in 1950 and its first use in a patient with manic symptoms two years later heralded the advent of a group of compounds whose main effect was thought to be exerted through blockade of DRD2 in the mesolimbic pathway. First generation antipsychotics were butyrophenones, such as haloperidol and droperidol; phenothiazines, such as chlorpromazine, thioridazine, fluphenazine, trifluoperazine and promethazine; and thioxanthenes such as zuclopenthixol, clopenthixol and flupenthixol. These compounds exert their action in the mesocortical pathway (involved in motivation and emotional responses), in the nigrostriatal pathway (involved in production of voluntary movement) and in the tuberoinfundibular pathway (involved in the production and secretion of prolactin).

Although the mechanism by which these drugs exert their therapeutic effect is still largely unknown (Karam *et al.* 2010), the DRD2 is coupled to $G_{i/o}$ proteins to inhibit adenylate cyclase (AC) and to modulate voltage-gated K^+ and Ca^{2+} channels. In addition, they cause troublesome side effects such as lethargy, anergia, dystonia, akathisia, rigidity, tremor, tardive dyskinesia and hyperprolactinemia.

Second generation antipsychotics, also called atypical due to their apparent absence of extra-pyramidal side-effects, were introduced from 1970 onwards. These are clozapine (CLO),

olanzapine (OLA), risperidone (RIS), quetiapine, ziprasidone, amisulpride, zotepine, sertindole and paliperidone. These compounds block DA D2 receptors and also serotonin receptors ($5HT_{2A}$ and $5HT_{2C}$), α-adrenoreceptors, hystamine H1 receptors and muscarinic acetylcholine receptors, and are credited with alleviating not only positive symptomatology but also some of the negative symptoms of SCZ (Murphy *et al.* 2006). Aripiprazole (ARI), a dual dopamine autoreceptor agonist and postsynaptic D2 receptor antagonist is the first of the so called third generation of antipsychotic medication (Ozdemir *et al.* 2002)**.**

The use of allosteric enhancers of the NMDA receptor such as glycine, D-serine and D-alanine has been tried with varied results (Buchanan *et al.* 2007), and GABA transmission enhancers such as MK-0777 and baclofen have also been tried (Karam *et al.* 2010). Similarly, the cholinergic system has also been the focus of recent interest as α7-nicotinic receptors and M1 muscarinic receptors are reduced in cortical areas of patients with SCZ and are likely to be involved in the cognitive deficits observed in this patient population (Karam *et al.* 2010).

Certain agents, like clozapine and ARI that are partial agonists of 5-HT(1A) receptors appear to have neuroprotective effects against excitotoxic injury in vivo (Cosi *et al.* 2005).In addition, antipsychotics increase striatal metabolism by normalizing mitochondrial function (Kung and Roberts, 1999) and increase the expression of ionotropic glutamatergic receptors (Heresco-Levy, 2003; Tascedda *et al.* 1999). And while clozapine (Pedrini *et al.* 2011) and risperidone increase BDNF (Lee and Kim, 2009), haloperidol decreases it (Pillai *et al.* 2008).

Antipsychotics have also other less known actions including effects on microglia and have a balancing effect on cytokines (Watanabe *et al.* 2010). An increase on IFN-γ, IL2 and TNF-α production following treatment with antipsychotics and a functional decrease of IL6 has been observed (Monji *et al.* 2009).

Therefore, it is reasonable to assume that the antipsychotic effect of these compounds could be mediated by more than just NT receptor blockade.

1.1.12. Neurodevelopment and neurodegeneration in schizophrenia

The neurodevelopmental hypothesis of SCZ (Murray and Lewis, 1987; Weinberger, 1987) purports that changes to the developmental component in cerebral circuitry are ultimately responsible for the onset of symptoms that subsequently occur around the third decade of life in most patients. According to Weinberberg, *"Schizophrenia is related to a defect in brain development. This defect predisposes to a characteristic pattern of brain malfunction in early adult life and to symptoms that respond to antidopaminergic drugs."* (Weinberger, 1996)

Although there is no conclusive evidence of developmental neuropathology associated with schizophrenia, traditionally, this theory is supported by the absence of detectable signs of brain damage such as widespread gliosis or neuronal loss, the observed presence of prodromal symptomatology, and the apparent absence of progressive and specific biochemical and molecular changes altering observable cellular processes which are present in traditionally viewed degenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD) or Parkinson's disease (PD) (Lieberman, 1999).

Indirect support to the neurodevelopmental hypothesis is weakly given by the presence of minor physical abnormalities (Green *et al.* 1989), reported evidence of neuropsychological abnormalities of children with schizophrenic mothers (Asarnow, 1988), but most importantly from two British birth cohorts epidemiological studies (Done *et al.* 1994; Jones *et al.* 1994) which showed that cognitive and social deficits as well as delayed neuromotor milestones during childhood were associated with a higher risk of developing schizophrenia in adulthood. Further evidence of neurodevelopmental abnormalities is given by the observed relationships between obstetric complications (Murray and Lewis, 1987) and prenatal viral exposure to influenza A2 virus during the second trimester (Mednick *et al.* 1988) and SCZ.

The most consistent evidence that SCZ is a disorder of the brain has been given by structural neuroimaging studies. Although, recent evidence suggests that antipsychotic treatment is partly responsible for reducing overall brain volume, and increasing ventricular or fluid volume (Moncrieff and Leo, 2010). These studies have shown volume reductions in amygdala, hippocampus, temporal and frontal cortices, enlargement of ventricles and temporal lobe hemisphere asymmetries which, albeit present at the initial stages of the disorder, appear to become more prominent as the illness progresses and its associated symptoms worsen and became chronic (Lieberman, 1999).

Nonetheless, it appears that there might be signs of astrocytosis and microgliosis present in the brains of people with SCZ (Schnieder and Dwork, 2011) . Other aspects of the disorder, such as the delayed onset, the progression of cognitive dysfunction observed in almost a third of the patients, the improvement observed with antipsychotic medication and the relationship between outcome and duration of untreated psychosis also signal the presence of a progressively worsening component in at least a subset of patients.

At a cellular level, a progression in neuronal atrophy, decreased neuropil and alterations in neuronal density have been reported in the anterior cingulated cortex, nucleus accumbens, hippocampus and thalamus (Perez-Neri *et al.* 2006), suggesting graded apoptosis,

probably as a result of excessive glutamatergic stimulation and its subsequent caspase activation (Glantz *et al.* 2006).

Glutamatergic hypofunction could be related to neurotoxicity (Chang *et al.* 2007) and hyperdopaminergic function (Halberstadt, 1995) as a result of loss of modulation. Chronic blockade of the NMDA receptors, which glutamatergic neurons use to stimulate GABAergic interneurons' inhibition of pyramidal neurons in the frontal cortex, leads to a decrease in GABAergic transmission and an increase in dopamine release that eventually leads to neurodegeneration though excessive glutamate release (Perez-Neri *et al.* 2006).

This is similar to the process seen following administration of ketamine and one that can be reversed with GABAergic agonists. In addition, NMDA receptor blockade by PCP reduced the phosphorylation of Akt and GSK-3β, decreasing the activity of the former and increasing the activity of the latter and inducing neurotoxicity during development; while depletion of GSK-3β by si-RNA attenuated caspase-3 activity blocked PCP-induced neurotoxicity (Lei *et al.* 2008). NMDA Receptor activation, on the other hand, results in the production of Nitric Oxide (NO) by the enzyme NO synthase (NOS). NO reduces dopamine reuptake and contributes to the hyperdopaminergic state and is also associated with glutamate neurotoxicity (Perez-Neri *et al.* 2006).

Another molecule regulated by glutamatergic transmission is brain-derived neurotrophic factor (BDNF), a neurotrophin with neuroprotective properties. Reduced expression of BDNF and its receptor are likely to increase vulnerability to cell death (Perez-Neri *et al.* 2006).

Lastly, deficiencies in energetic metabolism, such as oxidative stress and mitochondrial dysfunction have been reported in SCZ (Rezin *et al.* 2009; Scaglia, 2010; Wood *et al.* 2009). These alterations include mitochondrial hypoplasia, impairment in the phosphorylation system and altered mitochondrial-related gene expression (Ben-Shachar, 2009). Excessive NO concentrations are known to inhibit the mitochondrial respiratory chain.

Moreover, dopamine inhibits mitochondrial respiration by over stimulating phospholipase C-related intracellular calcium release in the brain and inducing cortical neuron apoptosis (Zhang *et al.* 2010a).

These two components, the neurodevelopmental and the neurodegenerative one, are not mutually exclusive and the course of the disorder is likely to be due to the interaction of both (Figure 1). Following the onset of frank psychosis (and usually florid positive symptoms) in late adolescence/early adulthood, SCZ often runs a progressively deteriorating course with the majority of patients manifesting an increased severity and persistence of negative symptoms,

cognitive deficits, social and functional impairment as the illness progresses from the early to the late stages.

Therefore, it appears that molecular mechanisms implicated in the pathophysiology of SCZ are consistent with a progressive neurodegenerative process, which origin is almost certainly neurodevelopmental and hence, it is reasonable to assume that peripheral makers for such processes exist and could be detected.

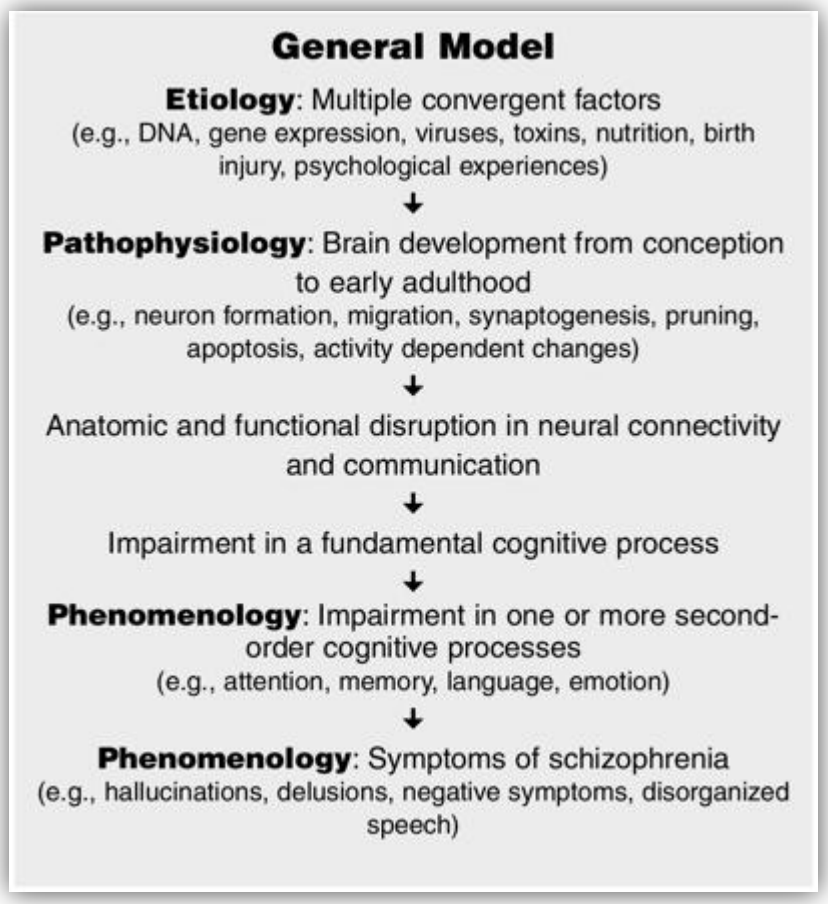

Figure 1. A general model integrating the concepts of schizophrenia.

1.2. Biomarker

1.2.1. Definition and use

A biomarker can be defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' (Biomarkers Definition Working Group, 2001) and following this, classified as Type 0: Natural history marker; Type 1: Biological activity marker and Type 2: Single or multiple marker(s) of therapeutic efficacy (Mildvan *et al.* 1997). In clinical trials terminology, a biomarker that substitutes for a clinical measure is a surrogate marker (Prentice, 1989; Rolan, 1997) and these are extremely valuable in medicine. Only a few have been proven to be reliable enough to be routinely used in clinical practice such as CD4 counts as an outcome measure in HIV/AIDS.

The potential clinical uses of biomarkers are many, and include facilitating objective and/or early diagnosis, monitoring disease process, determining staging and prognosis, and perhaps most challengingly, predicting treatment response.

The use of biomarkers in clinical trials to monitor outcome of therapy has huge potential to reduce the variability of clinical observations and in so doing increase the power of trials, reduce their costs and potentially facilitate the discovery of new medicines. Indeed two important processes designed to speed delivery of new therapeutics - the European Innovative Medicines Initiative (http://www.imi-europe.org/) and the US Critical Path initiative (http://www.c-path.org/) - rest very heavily on biomarkers.

The ideal biomarker for neurodegenerative diseases must have certain specific characteristics:

1. Accessible tissue.
2. Measurements are easy to quantify, reliable and quick, and reproducible.
3. Diagnostic biomarker should have small variation in the general population.
4. Unaffected by factors not associated with the disorder under study.
5. The biomarker changes linearly (either negatively or positively) with disease progression and in response to a disease modifying therapeutic intervention.

It is unlikely that any one biomarker will fulfil all these characteristics, and it is likely that the combination of clinical assessments, neuroimaging and biochemical profiling will be needed.

Proteomics are currently being employed to identify unique protein profiles in Alzheimer's disease (Ho *et al.* 2005), Parkinson's disease (Johnson *et al.* 2005), Huntington's disease (Zabel and Klose, 2004) and ALS (Poon *et al.* 2005) . Accumulating data will hopefully aid in identifying neurodegeneration-associated changes in protein expression that will ultimately be used as biomarkers (Henley *et al.* 2005).

In neuropsychiatric disorders, the most promising biochemical markers to date have been found through the analysis of CSF of AD patients. A decrease in the beta-amyloid1-42 peptide has been reported in 80–90% of Alzheimer's disease patients and the decrease is associated with APOE epsilon 4 allele (Sunderland *et al.* 2003; Sunderland *et al.* 2004), suggesting that beta-amyloid1-42 might represent an early biomarker of AD.

CSF total tau protein has been shown to be dramatically increased in Alzheimer's disease (Sunderland *et al.* 2003). The measurement of beta-amyloid1-42 peptide and total tau together is reported to have a sensitivity of around 90% in Alzheimer's disease diagnosis, which in combination with clinical evaluation and imaging ensures high accuracy of the clinical diagnosis (Blennow, 2004).

In contrast to the findings in Alzheimer's disease, CSF profiling in patients with frontotemporal dementia (FTD) reveals significantly decreased levels of CSF tau, which correlates significantly with frontal and temporal atrophy on volumetric imaging (Grossman *et al.* 2005).

An interesting potential CSF biomarker for Huntington's disease is orexin, which was shown to have a 72% reduction in CSF of Huntington's disease transgenic mice compared with wild-type littermates (Petersen *et al.* 2005), suggesting that orexin could be used as a biomarker reflecting neurodegeneration. Significant atrophy and loss of orexin neurons in Huntington's disease patients and atrophy and loss of orexin neurons in the lateral hypothalamus of Huntington's disease transgenic mice was also described in the same study.

1.2.2. Molecular biomarker discovery approaches in schizophrenia

Molecular biomarker discovery involves the analysis of biological samples. These might be tissue biopsies and extracts from the organ or system under study, or bodily fluids such as CSF, blood, urine, tears or saliva. Psychiatric illnesses pose the obvious problem that the organ of interest is not directly accessible and thus, CSF, as a fluid in direct contact with the brain, is intuitively the most appropriate peripheral tissue to look for biomarkers in psychiatry. However, sampling it from schizophrenic patients poses its own challenges. On the other hand, blood has considerable potential as a tissue for biomarker discovery as it is routinely collected

and represents a complex and dynamic tissue for gene, protein and metabolite studies. Some evidence suggests that in some cases, changes in blood reflect changes taking place in the brain, suggesting that blood based biomarkers might have some utility in brain disorders (Prabakaran, Wengenroth et al. 2007).

Conceptually, blood based markers of brain disease might occur in two ways. Firstly protein, metabolite or other molecules may cross from brain to blood. There is considerable interchange between CSF and blood and in some diseases the blood brain barrier may be disrupted, facilitating the substance exchange. Secondly, blood may reflect a signal of disease that is present systemically but only results in pathology causing symptoms in the brain. Either way, a marker that could be used as a biomarker in psychiatric illness may be present peripherally and could potentially be detected using the appropriate techniques.

Other challenges for SCZ research include the heterogeneity of the disorder at nosological and molecular level (Craddock *et al.* 2006), the complex interplay taking place in the cell between already altered at baseline cell signalling pathways and environmental insults; the effects of long term medication and the inherent limitations of current research technologies (Schwarz and Bahn, 2008).

Broadly speaking, there are two general approaches to molecular biomarker discovery. The most effective method to date has been a candidate approach – measuring molecules known to be associated with disease. Most of the examples of biomarkers in use in the clinic were derived from candidate studies; cholesterol as a biomarker predictive of cardiovascular disease, various hormonal markers reflecting germ cell tumour activity are examples of candidates that have been confirmed in large scale studies and are in clinical use. However, it is likely that there are many other markers of disease that cannot be predicted from knowledge of the pathology and may be discovered by the group of technologies variously known as data-driven approaches, unbiased technologies or, more prosaically, the 'omics'. These include genomics for study of DNA variants, transcriptomics for mRNA, metabolomics for products of metabolism and proteomics for peptides and proteins. In the following sections, we will briefly describe each one of those and expand on the latter.

Whilst for the most part the genome is a static, 'trait' measure (epigenetic changes apart), the transcriptome, metabolome and proteome (Schwarz and Bahn, 2008) are all dynamic, reflecting the changing needs of a cell or organ, disease status, and response to environmental stimuli. The transcriptome, metabolome and proteome can all be used to identify 'state' biomarkers, i.e. biomarkers that can be used to diagnose disease, to measure disease progression and/or as markers of the effectiveness of therapeutic intervention.

However, this ability to respond dynamically to changing conditions also provides a challenge to researchers as experimental variation (e.g. diet, pre-existing conditions, time of sampling, sample storage and handling) can itself affect the composition of the sample. Standardised protocols are therefore of critical importance to control for and minimise these effects.

1.2.3. Genomics

The genome is the complete set of DNA carried by an organism, and virtually every cell in the body carries a complete copy. As such, the role of genomics in the discovery of biomarkers is aimed at determining if there is a genetic predisposition to disease (i.e. a 'trait' biomarker). Whole genome studies typically use arrays which measure many variants in most genes (Cichon *et al.* 2009).

The technology is advancing rapidly with the number of variants increasing and the types of variants – single nucleotide polymorphisms, copy number variants, deletions, duplications, expansions and others all coming under increasing scrutiny and are helping to redefine the clinical phenotype (Cichon *et al.* 2009; Craddock *et al.* 2009; Fanous *et al.* 2007; Fanous and Kendler, 2008; O'Donovan *et al.* 2009). The cost of reading the entire genome is decreasing and at some point it is expected that this will become feasible for clinical studies. Nonetheless, this will be counterbalanced by the exponential complexity of data generated by such studies and by the costs of handling and analysing this data becoming singularly large.

1.2.4. Transcriptomics

The transcriptome is a set of mRNA (transcripts) that reflects genes that are expressed by a particular cell type. Like the genome, the transcriptome is most often studied using microarrays, and the technology is also advancing rapidly. First generation expression arrays were used to detect relative expression of gene transcripts by comparing the transcriptome of normal individuals with that of diseased individuals, for example. Increasingly, expression arrays are being used to estimate not just the total expression of a given gene but also to distinguish between differently expressed isoforms generated by alternative splicing. Many genes have multiple splice variants, and this seems to be a process of particular relevance in the brain, perhaps reflecting the generation of functional complexity from a relatively simple set of genes (i.e. not many more than simple organisms) (Calarco *et al.* 2007; Pan *et al.* 2008). Gene expression changes are generally validated using a separate method, ideally quantitative reverse transcription-polymerase chain reaction (RT-PCR).

In SCZ, these methods have already been fruitful when applied to brain tissue (Dean *et al.* 2009; Mitkus *et al.* 2008; Mudge *et al.* 2008; Perkins *et al.* 2007; Saetre *et al.* 2007) and in examining peripheral blood for biomarkers of the disorder (Chagnon *et al.* 2008; Ilani *et al.* 2001; Nadri *et al.* 2002; Numata *et al.* 2007; Perl *et al.* 2003; Zhang *et al.* 2008).

1.2.5. Metabolomics

Metabolomics (Goodacre, 2005; Nobeli and Thornton, 2006) refers to the analysis of metabolites, including amino acids, lipids, nucleotides and sugars, from tissues and body fluids. The metabolome is downstream of the genome, transcriptome and proteome, and as such may reflect more closely cell activity at a functional level (Allen *et al.* 2003; Nobeli and Thornton, 2006). Lipidomics is the part of metabolomics that refers to the analysis of the specific metabolite family, the lipids (Wenk, 2005). Like proteomics, no single technique is capable of analysing all metabolites due to the large dynamic range of metabolite concentrations and sample complexity (Nobeli and Thornton, 2006).

Two main technologies, Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectroscopy are currently in use for analysis of the metabolome, often in conjunction with various chromatographic techniques (typically gas chromatography (GC) for non-polar compounds, and Liquid Chromatography (LC) for polar compounds) to initially separate metabolite subsets. NMR spectroscopy can simultaneously measure different subsets of metabolites, although the NMR spectra associated with complex samples can be difficult to interpret. However, compared to MS, NMR spectroscopy has only moderate sensitivity, and is only able to detect the most abundant metabolites in plasma.

There have been few studies of the metabolome in SCZ although the data presented thus far is very promising. For example, the CSF of 152 drug naïve patients with first onset SCZ was compared to healthy controls initially and to the subsequently treated same patient group using ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR) (Holmes *et al.* 2006). The authors identified higher glucose levels and lower acetate and lactate concentration in the CSF of drug naïve patients when compared to controls. No difference in glucose plasma levels was observed, suggesting a CSF or brain specific alteration. Moreover, treatment with atypical antipsychotics for an average of 9 days restored the altered glucose levels to normality in 50% of patients whereas treatment with a typical agent it did not (the authors are cautious interpreting this result as only 6 patients were treated with a typical antipsychotic medication). Intriguingly, patients presenting with relapse as opposed to first episode failed to return their metabolome to normal following treatment.

In another study by the same group, drug naïve paranoid SCZ patients and prodromal patients were studied and compared to healthy controls (Huang *et al.* 2007) using ^{1}H-NMR and SELDI mass spectrometry. It was observed that 36% of prodromal patients showed proteomic abnormalities similar to the diseased group and 29% of them also shared similar metabolic profile. Unsurprisingly, only 29% of the group converted to the disorder within the three year follow up suggesting that the signature identified cannot be used to predict clinical outcome.

The CSF lipidome has also been the subject of study in recent years (Fonteh *et al.* 2006), and a lipidomic platform was successfully used to detect drug-specific changes in patients with SCZ treated with olanzapine, risperidone or aripiprazole (Kaddurah-Daouk *et al.* 2007) showing that levels of two major phospholipid classes, phosphatidylethanolamine and phosphotidylcholine were significantly downregulated in SCZ and that both olanzapine and risperidone, but not aripiprazole, increased concentrations of triacylglycerols and decreased free fatty acids levels.

^{1}H-NMR was also employed to study the lipid profile of monozygotic twins discordant for SCZ which showed alterations in both affected and unaffected subjects which could be correlated with Global Assessment of Function (GAF) scores but only in the affected females of the sample (Tsang *et al.* 2006).

1.2.6. Proteomics

The proteome refers to the peptide and protein complement of a particular tissue, cell or fluid at a given time and also describes post-translational modifications that can affect protein activity and location. Challenges associated with studying the proteome include the large dynamic range of proteins and peptides, as well as the complexity (proteins differ in their size and charge) and number of proteins in the sample; therefore no one technique can be used to study the entire proteome. Nonetheless, proteomic approaches aim to identify changes in protein expression and/or modification and are constantly being developed to increase coverage of the proteome.

a. Experimental design for proteomic studies

There are many different approaches to proteomics, which differs from genomics and transcriptomics in that even using multiple methods, only a portion of the true proteome in any complex sample is currently accessible to analysis. For this reason candidate approaches will have considerable utility for the foreseeable future – both individual protein estimates and large scale multiplex analyses.

For the most part these are variants of immune-based detection but other candidate protein multiplexing methods including mass spectrometry based approaches such as multiple reaction monitoring are increasingly used. Multiplexing in fluid phase using antibodies bound to colour coded beads allows up to 100 analytes to be measured in a single sample meaning that using this, and other analogous solid phase multiplexing approaches, candidate protein arrays can measure very large numbers of proteins (Olsson *et al.* 2005).

Experimental design in proteomics is in most cases a two-step process involving a discovery and a validation phase (Figure 2). There are a number of different proteomic methodologies available for biomarker studies which can be divided in biased and unbiased techniques. Unbiased techniques are used in the discovery stages of proteomic research for separation of analytes and to search for potential changes in any protein to be identified. These methods include: 2DPAGE and Liquid chromatography (LC). The biased techniques are used in the subsequent validation steps include all the affinity-binding methods such as Western blot, Enzyme-Linked ImmunoSorbent Assay (ELISA) and protein microarrays, which allows for in-depth profiling study on preselected proteins.

Analysis of large-scale proteomics data sets are usually complex and require careful planning of the most appropriate experimental design. The 2DPAGE workflow consists of the following steps:

1. Designing of the experiment.
2. Performing the experiment: Protein isolation, running the gel and scanning of the images.
3. Quantitation of the image: Background subtraction, spot segmentation and spot matching.
4. Analysis of the data: Normalisation and test for differences.
5. Interpretation and confirmation of results.

Statistical and computational methods are crucially involved at every level except in step 2.

A careful experimental design is necessary in order to reduce the complexity of the research question, to minimise non-treatment variability, to maximise estimation accuracy, to reduce the number of samples needed (reducing time and cost) and to chose the more cost-effective platform or technology with which the experiments can be conducted. Adequate experimental designs are those that define the key hypothesis and identify the crucial variables to test, identify the most efficient experimental design, devise systems to identify and mitigate sources of variability, define statistical approaches to maximise power under

reasonable assumptions and estimate the required sample size based on effect size, variability, and desired false positive and false negative rates.

Sources of variability could be found:

1. At a biological level: Gender, diet, age, ethnicity, environmental factors, and genetic make-up.
2. At a technical level: Sample quality, experimenter, materials and measurement errors.
3. At systems level: Platform used, instrumentation, settings for analysis (spot detection, matching and normalisation).

Intersubject variability can be controlled by using a longitudinal design by which each subject acts as a control for himself prior to developing the condition under study or by carefully matching of known parameters such as gender, age, ethnicity, and other known environmental factors. The former is extremely difficult to achieve, particularly in neuropsychiatric studios and therefore, in the human sample selected for study in this thesis, subjects were matched for age, gender, ethnicity, alcohol, tobacco and cannabis use. Since atypical antipsychotic medication has been linked with the onset of metabolic syndrome and in order to minimise the effects of medication, which can also induce protein expression changes, cases were grouped in three subsets as follows: subjects treated with Olanzapine, subjects treated with Risperidone and subjects who were medication free at the time of sampling.

All experiments presented in this thesis were performed by the author using the same standard protocol and each batch included an equal number of cases and controls to minimise the so-called ‘batch effect’.

Analysis and interpretation of the data was performed using the same specialised software (Progenesis SameSpots) in conjunction with a biostatistician and an experienced postdoctoral researcher.

Confirmation of results was obtained using an independent sample and a different technique than during the discovery phase.

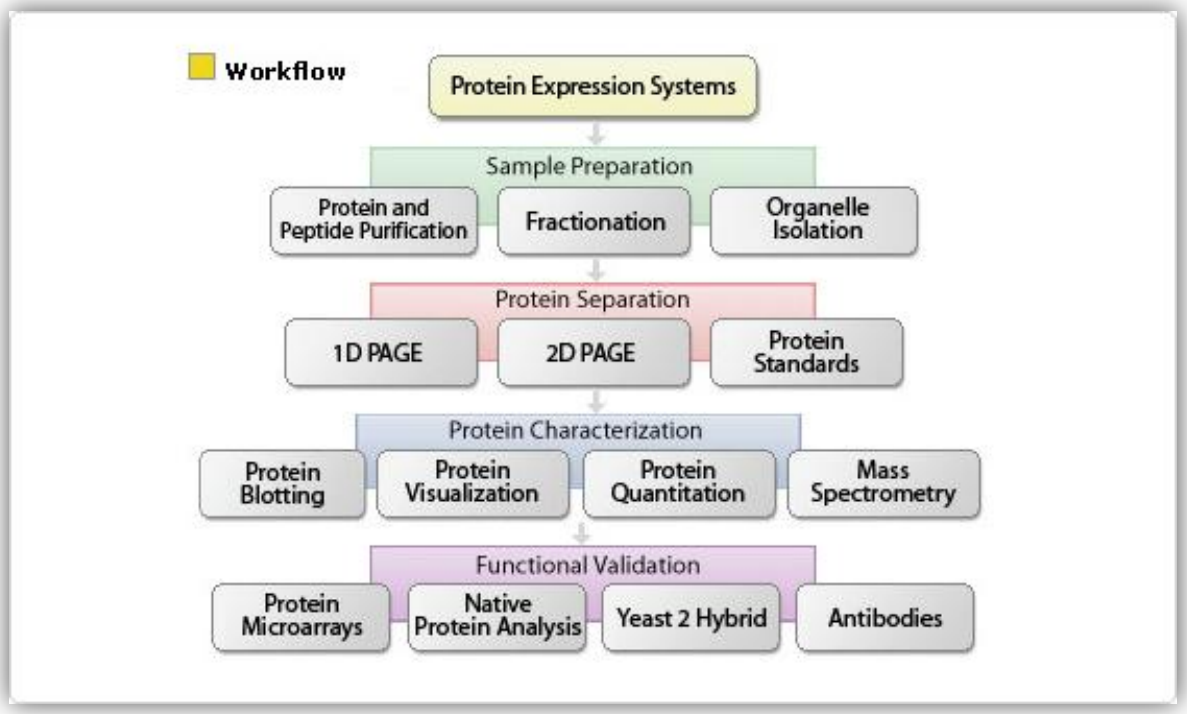

Figure 2. Proteomic workflow (Invitrogen website).

b. Sample preparation

Sample preparation is a key step in proteomic research and standardised protocols should be followed for sample capture and extraction, transportation, storage and handling to minimise experimental error and ensure reproducibility of results as proteins are easily degraded. Isolated cells, whole tissues or body fluids are the usual sources of protein samples, which are found amongst other molecules and cellular components.

Proteins are very heterogeneous in terms of size, charge, solubility and structure and successful protein separation depends greatly on sample preparation and processing, which have to be adapted to the aim of the experiment and its target analytes.

The main sources of variation in proteomic experiments occur during the pre-analytical stage and account for over 90% of the total experimental errors. In addition, hundreds, if not thousands of samples, are needed for biomarker discovery experiments in clinical populations. For these reasons and in order to standardize the analytical process prior to deciding which techniques to use for the experimental design, it is paramount to consider:

1. The clinical population under study and the biological variation due to gender, ethnicity, age, lifestyle and medication, among others, that surrounds the diseased subjects and its appropriately matched control sample. Exhaustive phenotypic data should be collected to fully characterize the population which the sample is derived from. This should include as many clinical and demographic variables as it is feasible to collect, as the existence of unknown molecular sub-phenotypes underlying similar clinical presentations is highly likely to exist in psychiatric populations.
2. The origin of the sample. This will mainly be biopsy, bodily fluids or post-mortem tissue.
3. The collection of the sample. In the case of blood-based experiments, a different subset of proteins will be obtained according to whether EDTA or citrate coated tubes have been used.
4. The period of time elapsed between sample collection and processing, including the length and the temperature in which the sample was kept.
5. The processing of the sample, including centrifugation speed, time and temperature; volume and composition (plasma, serum, RBC, WBC pellet) of aliquots (if body fluids) and homogenization (if solid tissue) or cell culture protocols followed.
6. The storage of the sample with regards to temperature in which the sample is to be kept (-80°C for best preservation) and number of freeze-thaw cycles.
7. The amount of sample needed to conduct the experiments.

8. Other sample dependent aspects, such as enrichment or depletion strategies. In novel disease-related biomarker discovery techniques in body fluids, for low-abundance proteins, it is reasonable to consider depletion of highly abundant proteins such as albumin or immunoglobulins when the target protein is not-known. Depleted samples are less intricate in terms of composition and may offer an advantage for traditional separation proteomic techniques such as 2DPAGE. The main disadvantages of depletion is that potential targets can be depleted inadvertently and that, since 90% of the protein content in plasma can be attributed to the nine most abundant human serum proteins (albumin, IgG, haptoglobin, transferring, transthyretin, α_1-antitrypsin, α_1-acid glycoprotein, hemopexin and α_2-macroglobulin) (Righetti *et al.* 2005), depleted plasma samples could easily fall below the levels of detection of current proteomic separation technologies. Enrichment strategies, such as subcellular fractionation, are not as common as the target protein should be known a priori, although they are of tremendous importance to elucidate the molecular basis of biological processes.

c. Discovery phase

This is a hypothesis-generating exercise which main aim is to examine the proteome as comprehensibly as possible in order to identify protein expression changes within the diseased (or treated) and the control groups. This is a particularly challenging effort since protein expression encompasses 10 to 12 orders of magnitude in bodily fluids, which makes the task of examining the proteome using a single technique an impractical task.

Non-biased techniques such as two-dimensional gel electrophoresis and liquid chromatography are usually employed at this stage. The ideal outcome is to identify a limited number of potential candidates that are subsequently identified by employing MS-based techniques.

d. Validation

In the validation stage, the potential targets (analytes) need to be successfully identified in an independent sample using an alternative method of detection. The validation stage is no longer a hypothesis-generating step, but a hypothesis-testing one in which the search for analytes is guided and specific.

This step involves the use of pre-existing antibodies and fluorescent probes targeted against epitopes of the analytes of interest. Antibody-based assays such as Western blot and ELISA are the preferred methods as hundreds of samples can be tested in a relatively short period of time.

Validation has to be performed at least in duplicate and is difficult due to several factors:

1. Clinical sample factors: Finding an independent sample in which to replicate the findings is difficult as inclusion and exclusion criteria may vary between studies and different populations might be exposed to differential environmental agents that alter protein expression. A priori power calculations need to be performed to determine the number of cases and controls required for validation. In addition, larger samples are needed for validation (a few hundreds) than for discovery and thus, the matching between cases and controls for potential factors altering gene expression, such as age, gender, ethnicity, smoking status, alcohol use, lifestyle and diet are less likely to be as well matched.
2. Biological sample processing, storage and preparation; In the case of blood-based biomarkers, factors such as the needle gauge used in venipuncture, the additives in the collecting tubes (i.e. citrate, ethylenediaminetetraacetic acid (EDTA)), the time between collection and processing and the temperature in which the tube is kept while transporting the sample from the place of extraction to the processing lab, the speed and time of centrifugation to obtain plasma or serum from whole blood, the storage temperature (ideally -80˚C) and the number of freeze-thaw cycles affect the activity of the proteases contained and samples are found in different stages of protein degradation.
3. Assay factors: Specific antibodies have to be found commercially for the analyte under study. The selected antibody has to be able to recognize an epitope presented by the protein tertiary structure.

e. Core proteomic techniques

1. Two-dimensional polyacrylamide gel electrophoresis (2DPAGE)

Unbiased techniques are those used to search for potential changes in any protein present in the sample. Perhaps the best established is 2-dimensional gel electrophoresis (2DPAGE) (O'Farrell, 1975; Righetti *et al.* 2005) coupled with mass spectrometry (MS). 2DPAGE separates proteins by isoelectric charge and molecular weight, and is capable of separating different isoforms and post-translational modifications of the same protein. A variant of this technique is the 2-dimensional differential gel electrophoresis (DIGE), in which two or three differentially pre-labelled samples are run in the same gel (Shaw *et al.* 2003; Tonge *et al.* 2001; Van den and Arckens, 2004).

2. Liquid chromatography (LC)

LC is a complementary approach to gel-based proteomic techniques. In High –Performance (or Pressure) Liquid Chromatography (HPLC) a pump, instead of gravity alone, is commonly used to move the liquid analyte or mobile phase through a column containing particles called the stationary phase which slows down the progress of the solution using specific chemical or physical interactions.

The retention time is the time that it takes for a given mobile phase to elute (pass through a specific stationary phase) and it is considered to be a unique identifier of the sample. Thus protein separation can be obtained according to charge, using Ion Exchange Chromatography (IEX); according to size by employing gel filtration columns; according to hydrophobicity, using Reversed Phase Chromatography (RPC); and biorecognition through the use of Affinity Chromatography (AC).

IEX is the first choice as it can be used with any sample regardless of its amount and concentration and can be performed under native or denatured conditions. Gel filtration is the easiest LC to perform but can only be used with low volumes and has low resolution due to its diluting rather than concentrating nature.

RPC is capable, on the other hand, of concentrating diluted samples and can also be used to purify samples from salts and urea in a step prior to separation, which makes it an ideal choice for samples with high concentrations of salts or other additives. AC relies on the use of commercial and self-made ligands to specifically remove and isolate the target analyte (usually a protein considered not to be of interest or too abundant).

3. Matrix-Assisted Laser Desorption/Ionisation (MALDI)

Following 2DGE, MS is used to determine protein identity of spots that differ between samples. The spot is subjected to in-gel enzymatic digestion and protein fragments are separated according to their mass-to-charge ratios (m/z). This is achieved by ionising the digested analyte molecules using Matrix-Assisted Laser Desorption/Ionisation (MALDI) (Hortin, 2006) or Electrospray Ionisation (ESI) (Cech and Enke, 2001; Fenn *et al.* 1989). The resulting gas-phase ions are detected using a mass analyser and the output of detected masses (called a peptide mass fingerprint) (Thiede *et al.* 2005) is compared to a database to determine the protein or peptide identity (Palagi *et al.* 2006).

Primary structure of the peptide can be determined using Mass Selection/Mass Separation (MS/MS) or tandem MS whereby a narrow m/z range is set on the mass spectrometer to enable selection of specific ions (Shevchenko *et al.* 1996). These ions are then fragmented and the amino acid sequence is determined from the fragment masses.

4. Quadrupole Time-of-Flight (Q-ToF) mass spectrometry

These instruments combine a mass-filtering quadrupole analyser and a collision cell with a non-scanning reflector ToF analyser, allowing the user to acquire MS/MS data with high mass accuracy, resolution and sensitivity. This instrument is usually coupled with HPLC and has become the instrument of choice for proteomics since its initial application for oligosaccharide analysis (Morris *et al.* 1996).

5. Surface-enhanced laser desorption/ionisation (SELDI) mass spectrometry

Another approach to separation that does not rely on gels, uses solid phase matrices to 'hold' analytes from within a complex mixture prior to MS. Surface-enhanced laser desorption/ionisation (SELDI) (Kiehntopf *et al.* 2007; von *et al.* 2001; Yip and Lomas, 2002) is a modification of MALDI whereby an additional step is introduced to retain proteins according to their biochemical properties (i.e. strong cationic, strong anionic, reverse phase, hydrophobic, metal affinity).

Retained proteins then undergo MALDI-MS and spectra from different samples are compared to determine their differences. This, unlike many of the approaches above, is a true high throughput approach but the output is a trace rather than a set of proteins. This trace may itself be a biomarker and different in controls and patients but it is difficult to see how this could be used in clinical practice and so most SELDI experimenters go on to identify the proteins and peptides resulting in trace differences between cases and controls.

6. Separation and identification: Gel-free approaches

Gel free mass spectrometry is increasingly applicable to biomarker studies for either hypothesis-driven discovery or validation stages. One approach is to label samples from different sets – patients and controls for example. Isotope-Coded Affinity Tag (ICAT) (Gygi *et al.* 1999), isobaric tags for relative and absolute quantification (iTRAQ) (Ross *et al.* 2004), Tandem Mass Tags (TMT) (Thompson *et al.* 2003a) and other reagents have been developed to label peptides in such samples.

ICAT isotopic heavy or light reagent labels the side chains of cysteinyl residues in reduced protein samples, whereas iTRAQ and TMT reagents chemically tag the N-terminal of peptides. Samples are then mixed together and analysed by MS; the ratio of the signal intensities of the tags is a reliable measure of the relative protein expressions in the original samples. Use of labelled internal standards enables quantitative data to be obtained.

Hydrophobic proteins are better represented using these gel-free approaches compared to 2DGE.

7. Western blots and ELISA

The Western blot is a technique used to identify proteins in complex mixtures such as tissue homogenates or body fluids. The sample is treated to denature the proteins contained and these are subsequently separated using 1-Dimensional gel electrophoresis (there is no separation based on isoelectric focusing). The proteins are transferred to a nitrocellulose membrane where they are incubated with a primary antibody targeted towards and epitope of the target protein. Following a period of incubation with this antibody, a secondary antibody against it and containing a fluorescent probe is added to the sample. The membrane is scanned and the protein levels are evaluated using densitometry or spectrophotometry.

Enzyme-linked immunoabsorbent assay (ELISA) uses 96-well microtiter plates with standardized and specific commercial antibodies in order to reduce experimental error, increase throughput and obtain semi-quantitative results using a standard curve.

8. Protein microarrays

Protein microarrays have evolved from the Western blot and ELISA techniques (also called “macroarrays”) following the incorporation of the basic technology advancements developed for genomics and transcriptomics studies. Microarrays use smaller sample volumes and allow for the characterisation of several biological events simultaneously with good reproducibility, high sensitivity and accuracy.

This multiplex approach enables the study of protein-protein interactions, the detection and quantification of substrates and products of enzymatic reactions, and the identification of transcription factors for protein activation, amongst others. The carrier is usually a bead or piece of glass or silicon containing capture molecules which are usually antibodies targeting the specific analyte(s) of interest from cell lysates solutions or biological fluids such as plasma, serum or CSF. Other capture molecules used in recent years are nucleic acids, receptor, enzymes and proteins or antigens that can be used as capture molecules when the targets are specific antibodies.

Label-based assays use specific tags against the target analyte to allow simultaneous detection following capture with immobilized antibodies although there is a risk of the tag interfering with the antigen-antibody interaction and suffer from limited specificity and sensitivity.

Sandwich assays use immobilized antibodies to capture unlabeled proteins that are detected following incubation with another antibody, and can have higher specificity and detection sensitivity than label-based assays, as only one sample can be incubated at a time and employ two antibodies per analyte which dramatically reduce background interference signal.

1.2.7. Proteomic studies in brain in schizophrenia

A recent review of the published quantitative neuroproteomic studies of SCZ examined 11 post-mortem proteomic investigations (Table 2), revealing that 16 (Table 3) grey matter and 8 (Table 4) white matter proteins were differentially expressed in the same direction in two or more of those studies.

Cellular assembly and organisation were particularly disrupted in both grey and white matter and the glycolysis-gluconeogenesis was the major signalling pathway altered in both (English *et al.* 2011).

Table 2. Summary of the 11 post-mortem brain proteomic investigations included in English et al. 2011.

Brain Region	Samples	Findings
PFC, BA10, gray matter	24 SCZ, 23 bipolar disorder, 19 major depression, and 23 controls	8 altered proteins, cytoskeletal and metabolism alterations
dlPFC, BA 9, gray matter and white matter	10 SCZ and 10 controls	215 altered proteins, metabolism and oxidative stress
ACC, BA24, gray matter	10 SCZ and 10 controls	42 altered proteins; metabolism, oxidative stress; cytoskeletal abnormalities
ACC, gray matter	15 SCZ, 15 bipolar disorder, 15 major depression, and 15 controls	35 altered proteins, cytoskeletal and metabolism alterations
dlPFC, BA 46, gray matter	17 SCZ, 20 bipolar disorder, and 20 controls	24 diagnosis biomarker peaks
dlPFC, BA 9, gray matter	34 SCZ, 32 bipolar disorder, and 30 controls	66 altered proteins: 18 proteins in SCZ, 55 proteins in bipolar disorder; metabolism and synaptic alterations
ACC, BA 24, white matter	10 SCZ and 10 controls	32 altered proteins; metabolism, cytoskeletal, and synaptic abnormalities
CC, genu	11 SCZ and 10 controls	64 altered proteins; cytoskeletal, signal transduction, and metabolism alterations
dlPFC, BA 9, gray matter, membrane microdomain fraction	10 SCZ, 10 bipolar disorder, and 10 controls	56 altered proteins by GeLC-MS/MS 96 altered proteins by 2D-DIGE; 16 proteins altered by both methods; synaptic, cytoskeletal, and metabolism alterations
IC, gray matter, layer 2	12 SCZ and 13 controls	57 altered proteins; neuronal plasticity, neuritic outgrowth, cytoskeletal stability
dlPFC, BA 9, white matter	35 SCZ, 33 bipolar disorder, and 35 controls	70 altered proteins: 41 proteins in SCZ, 29 in bipolar disorder; metabolism, signal transduction, cytoskeletal abnormalities

Table 3. The 16 candidate grey matter proteins altered in the same direction in two or more proteomic investigations of SCZ (English *et al.* 2011).

Protein Name	Symbol	Gene Locus	Swissprot Accession Number	Bipolar Disorder Associated	Parkinson's Disease Associated	Alzheimer's Disease Associated
Aldolase C, Fructose-Bisphosphate	ALDOC	17cen-q12	P09972	Yes	Yes	Yes
Creatine Kinase, Brain	CKB	14q32	P12277	Yes		Yes
Dynamin 1	DNM1	9q34	Q05193			
Dihydropyrimidinase-Like 2	DPYSL2	8p22-p21	Q16555	Yes		Yes
Enolase 1 (alpha)	ENO1	1p36.3–p36.2	P06733	Yes		Yes
Enolase 2 (gamma, neuronal)	ENO2	12p13	P09104	Yes		Yes
Glial Fibrillary Acidic Protein	GFAP	17q21	P14136	Yes	Yes	Yes
GLU-Ammonia Ligase	GLUL	1q31	P15104			Yes
Guanine Nucleotide Binding Protein, beta Polypeptide 1	GNB1	1p36.33	P62873	Yes	Yes	Yes
Internexin Neuronal Intermediate Filament Protein, Alpha	INA	10q24.33	Q16352	Yes	Yes	Yes
N-Ethylmaleimide-Sensitive Factor	NSF	17q21	P46459			
Phosphoglycerate Mutase 1	PGAM1	10q25.3	P18669		Yes	Yes
Peroxiredoxin 2	PRDX2	19p13.2	P32119	Yes	Yes	Yes
Septin 3	SEPT3	22q13.2	Q9UH03			
Triosephosphate Isomerase 1	TPI1	12p13	P60174	Yes		Yes
Ubiquitin Carboxyl-Terminal Esterase L1	UCH-L1	4p14	P09936	Yes	Yes	Yes

ACC, anterior cingulate cortex; BA, Brodmann's area; CC, corpus callosum; dlPFC, dorsolateral prefrontal cortex; IC, insular cortex.

Table 4. The 8 candidate white matter proteins altered in the same direction in two or more proteomic investigations of SCZ (English et al. 2011).

Protein Name	Symbol	Gene Locus	Swissprot Accession Number	Bipolar Disorder Associated	Parkinson's Disease Associated	Alzheimer's Disease Associated
Dihydropyrimidinase-like 2	DPYSL2	8p22-p21	Q16555	Yes		Yes
Inositol-Monophosphatase 1	IMPA1	8q21	P29218			
Neurofilament, Light Polypeptide	NEFL	8p21	P07196		Yes	
Parkinson Disease 7	PARK7	1p36.33	Q99497			Yes
14-3-3 zeta	YWHAZ	8q23.1	P63104			
L-Lactate Dehydrogenase Chain *B*	LDHB	12p12.2	P07195			Yes
Peroxiredoxin 2	PRDX2	19p13.2	P32119	Yes	Yes	Yes
Stathmin 1	STMN1	1p36.1	P16949		Yes	

ACC = anterior cingulate cortex; BA = Brodmann's area; CC = corpus callosum; dlPFC = dorsolateral prefrontal cortex.

1.2.8. Proteomic studies in cerebrospinal fluid in schizophrenia

A search on proteomic studies of CSF in SCZ (using the key words 'human', 'CSF', 'SCZ' and 'protein' in PubMed) retrieved 281 entries. The majority of the studies are performed using the 'classical' workflow of gel electrophoresis or liquid chromatography coupled to mass spectrometry.

Reportedly upregulated proteins found in the CSF of patients with SCZ were found to be:

1. Angiotensin I-converting enzyme (ACE) (1306 amino acids; 149715 Da) (Wahlbeck *et al.* 1993; Wahlbeck *et al.* 2000).
2. Neural cell adhesion molecule 1 (NCAM1) (858 amino acids; 94574 Da) (Poltorak *et al.* 1997; Vawter *et al.* 2001).
3. S100 calcium binding protein B (S100B) (92 amino acids; 10713 Da) (Rothermundt *et al.* 2004; Schmitt *et al.* 2005).
4. Neuron-specific enolase (ENO2) (434 amino acids; 47269 Da) (Li *et al.* 2006; Vermuyten *et al.* 1990).
5. IL6 (212 amino acids; 23718 Da) (Garver *et al.* 2003; van Kammen *et al.* 1999).
6. IL2 (153 amino acids; 17628 Da) (Licinio *et al.* 1993; McAllister *et al.* 1995).

These proteins are mainly involved in immune response (ACE, NCAM1, S100B, Il-6 and Il-2) and calcium signalling (NCAM1, S100B, ENO2, Il-2), although mechanisms involved in the modulation of dopamine, apoptosis, cell adhesion and proliferation, axon guidance, glucose metabolism also appear to be affected.

Proteins downregulated in the CSF of patients suffering from SCZ are:

1. Neurotensin (NTS) (170 amino acids; 19795 Da) (Binder *et al.* 2001; Widerlov *et al.* 1982).
2. Thyrotropin-releasing hormone (TRH) (242 amino acids; 27404 Da) (Banki *et al.* 1992; Sharma *et al.* 2001).
3. Transthyretin (TTR) (147 amino acids; 15887 Da) (Huang *et al.* 2006a; Wan *et al.* 2006).
4. Brain derived neurotrophic factor (BDNF) (247 amino acids; 27818 Da) (Issa *et al.* 2010; Pillai *et al.* 2010).
5. Cholecystokinin (CKK) (115 amino acids; 12669 Da) (Beinfeld and Garver, 1991; Garver *et al.* 1990).

The pathways highlighted as being potentially altered in SCZ by these analytes are dopamine and glutamatergic transmission, apoptosis, insulin secretion and nervous system development.

Neurotensin and cholecystokinin are brain and gastrointestinal neuropeptides which role in the CNS is not entirely ascertained but are thought to be involved in G-protein couple receptor signalling pathways and in modulation of dopaminergic and glutamatergic transmission in nigrostriatal and mesocorticolimbic pathways, and may have hypothermic and analgesic effects.

Thyrotropin-releasing hormone and transthyretin regulate thyroid function by regulating the biosynthesis of TSH in the anterior pituitary gland and acting as a neurotransmitter/ neuromodulator in the central and peripheral nervous systems in the case of the former and by probably transporting thyroxin from the bloodstream to the brain in the case of the latter.

Brain derived neurotrophic factor, promotes survival and differentiation of selected neuronal populations during development and participates in apoptosis, long term potentiation (LTP) and depression (LTD) and in axonal and dendritic modulation and plasticity. BDNF binds to Trk (tropomyosin-receptor-kinase) receptor B which is a single transmembrane catalytic receptor with intracellular tyrosine kinase activity and which ligand is usually Neurotrophin.

Proteins found in CSF which direction of expression (either up or downregulation) had been replicated in SCZ in at least two independent studies along with the references for the studies mentioned in this section are listed and summarised in Table 5.

Other proteins to consider as potential biomarkers for SCZ have been detected in the CSF of schizophrenic subjects but they have not been replicated to date. Additional evidence of their involvement in the pathological process of psychosis is given by evidence of their presence in other tissues and by their involvement in other neuropsychiatric disorders and are summarised in Table 6, where references are also provided.

Downregulated analytes are: Nerve growth factor (NGF) (241 amino acids; 26959 Da). NGF regulates sympathetic and sensory nervous systems and is involved in apoptosis, memory, inflammatory response and nerve growth. NGF binds to the receptor TrkA and participates in anti-apoptotic neuronal mechanisms and NT synthesis; glycogen synthase kinase 3 beta (GSK-3β) (420 amino acids; 46744 Da). GSK-3β is part of the Wnt signalling pathway and regulates glycogen synthase. Its activity is regulated by phosphorylation (Akt, protein kinase (PK) A, PKC, ribosomal S6 kinase (RSK) and ribosomal protein S6-p70-protein kinase (S6K) and

dephosphorylation (protein phosphatase (PP) 1 and 2A) and by binding to protein complexes (with beta catenin). It is thought to be involved in synaptic plasticity via regulation of the NMDA receptor trafficking; orexin A (hypocretin-1) (HCRT) (131 amino acids; 13363 Da) is a neuropeptide thought to influence food intake and regulate the sleep-wake cycle through hypothalamic G-protein-couple receptors (orexin/hcrt-1 hypocretin receptors, OX1 and OX2); and chromogranin A (parathyroid secretory protein 1) (CHGA) (457 amino acids; 50688 Da), a pancreastatin which inhibits glucose induced insulin release from the pancreas.

Upregulated proteins are: neurosecretory protein VGF (VGF) (615 amino acids; 67258 Da) which is upregulated by nerve growth factor and may be involved in regulation of cell-cell interactions during brain development; and the synaptosomal associated protein 25 (SNAP 25) (206 amino acids; 23315 Da) which has a role in synaptic transmission and regulation of insulin and glutamate secretion. Both analytes seem to be upregulated in CSF in a pilot study and a replication by the same authors (Huang *et al.* 2006a; Huang *et al.* 2007). High throughput proteomic techniques have been used to examine CSF (SELDI-TOF), liver (2D-DIGE), red blood cells (2D-DIGE) and serum (ELISA) in independent cohorts of patients with SCZ (Huang *et al.* 2008) consistently showing decreased levels of apolipoprotein A1.

Other candidates that are detectable but which expression can not be clearly determined in CSF are Neurogranin (NRGN) (8 amino acids; 7618 Da), which binds to calmodulin in the absence of calcium and exert its action during synaptic development as a third messenger substrate for PKC; Ras-related protein (RAB3) (220 amino acids; 24984 Da), which is involved in exocytosis and NT release; growth-associated protein 43 (Neuromodulin) (GAP43) (238 amino acids; 24803 Da), highly expressed in neuronal growth cones during axonal regeneration and growth and synaptotagmin (SYT1) (422 amino acids; 47573 Da) a membrane protein serving as Ca(2+) sensors for vesicular trafficking and exocytosis and NT release at synapses. The former three are downregulated in the brain of schizophrenic patients while the latter is upregulated (Davidsson *et al.* 1999).

Table 5. The main protein candidates from proteomic studies in human CSF in SCZ.

Protein Name	Swissprot Accession Number Gene Symbol Gene Locus	CSF in SCZ	Detected in CSF in other neuropsychiatric disorders	Main Cell processes involved
Angiotensin I-Converting Enzyme	P12821 ACE 17q23.3	**CSF (↑ in chronic patients)** (Wahlbeck *et al.* 1993) (Wahlbeck *et al.* 2000)	**Parkinson's CSF (↓)** (Zubenko *et al.* 1985) **Alzheimer's CSF (↓)** (Zubenko *et al.* 1985) (Miners *et al.* 2009)	Regulation of vasoconstriction/vasodilatation, hormone catabolic process, mononuclear cell proliferation, modulation of dopamine turn-over in brain
Neural cell adhesion molecule 1	P13591 NCAM1 11q23.1	**CSF (↑)** (Poltorak *et al.* 1997) (Vawter *et al.* 2001)	**Parkinson's CSF (↑)** (Yin *et al.* 2009) **Alzheimer's CSF (↑)** (Yin *et al.* 2009)	Axonogenesis and axon guidance, cell adhesion, cytokine-mediated signalling pathway, positive regulation of calcium-mediated signalling
S100 calcium binding protein B	P04271 S100B 21q22.3	**CSF (↑)** (Rothermundt *et al.* 2004) (Schmitt *et al.* 2005)	**Alzheimer's CSF (↑)** (Petzold *et al.* 2003)	Cell proliferation
Neuron-specific enolase (Gamma enolase, enolase 2)	P09104 NSE (ENO2) 12p13	**CSF (↑)** (Vermuyten *et al.* 1990) (Li *et al.* 2006)	**Alzheimer's CSF (↓)** (Cutler *et al.* 1986)	Glucose metabolism, response to oestradiol
Interleukin-6	P05231 IL6 7p21	**CSF (↑)** (van Kammen *et al.* 1999) (Garver *et al.* 2003)	**Parkinson's CSF (↑)** (Muller *et al.* 1998)	Acute phase immune response, bone remodelling, cell redox homeostasis, negative regulation of apoptosis
Interleukin-2	P60568 IL2 4q26-q27	**CSF (↑)** (Licinio *et al.* 1993) (McAllister *et al.* 1995)	**Parkinson's CSF (↑)** (Mogi *et al.* 1996)	T cell differentiation, anti-apoptosis, cell adhesion, elevation of cytosolic calcium ion concentration, negative regulation of: heart contraction, inflammatory response, lymphocyte proliferation, protein phosphorylation; positive regulation of: cell growth and proliferation, immunoglobulin secretion, phosphorylation and tissue remodelling

Table 5 (continued)

Protein Name	Swissprot Accession Number Gene Symbol Gene Locus	CSF in SCZ	Detected in CSF in other neuropsychiatric disorders	Main Cell processes involved
Neurotensin	P30990 NTS 12q21	**CSF (↓)** (Widerlov *et al.* 1982) (Binder *et al.* 2001)		Regulation of blood vessel size, signal transduction, modulates dopaminergic transmission and enhances GLU exotoxicity
Thyrotropin-Releasing Hormone	P20396 TRH 3q13.3-q21	**CSF (↓)** (Banki *et al.* 1992) (Sharma *et al.* 2001)	**Depression CSF (↑)** (Banki *et al.* 1988) **Alzheimer's CSF (↑)** (Pekary *et al.* 1991)	Negative regulation of GLU secretion, positive regulation of insulin secretion, response to ethanol, corticosterone and glucose stimulus, response to hypoxia
Transthyretin (Prealbumin)	P02766 TTR 18q12.1	**CSF (↓)** (Huang *et al.* 2006a) (Wan *et al.* 2006)	**Parkinson's CSF (↓)** (Abram *et al.* 1990)	Intra/extra cellular transport, hormone binding
Brain derived neurotrophic factor	P23560 BDNF 11p13	**CSF (↓)** (Pillai *et al.* 2010) (Issa *et al.* 2010)	**Parkinson's CSF (↑)** (Salehi and Mashayekhi, 2009) **Alzheimer's CSF (↓)** (Li *et al.* 2009)	Nervous system development
Cholecystokinin	P06307 CKK 3p22-p21.3	**CSF (↓)** (Garver *et al.* 1990) (Beinfeld and Garver, 1991)	**Bipolar Disorder CSF (↓)** (Verbanck *et al.* 1984)	Activation of PKC activity by G-protein couple receptor protein signalling pathway, axonogenesis, negative regulation of appetite, neuron migration, positive regulation of apoptosis, cell proliferation and mitochondrial depolarization, regulation of sensory perception of pain, release of cytochrome c from mitochondria, signal transduction

Table 6. Other protein candidates from proteomic studies in human CSF in SCZ.

Protein Name	Swissprot Accession Number Gene Symbol Gene Locus	SCZ	Bipolar Disorder	Parkinson's disease	Alzheimer's disease	Main Cell processes involved
Nerve Growth Factor	P01138 NGF 1p13.1	**CSF (↓)** (Kale *et al.* 2009) **Serum (↓)** (Xiong *et al.* 2010)	**Plasma (↓)** (Barbosa *et al.* 2010)	**Serum (↓)** (Lorigados *et al.* 2002)	**Hippocampus and CSF (↑)** (Hock *et al.* 2000a; Hock *et al.* 2000b; Mashayekhi and Salehin, 2006)	Apoptosis, inflammatory response, activation of phospholipase C, axonogenesis, response to electrical stimulus and to glucocorticoid stimulus
Glycogen synthase kinase 3 beta	Q6FI27 GSK3B 3q13.3	**CSF (↓)** (Kozlovsky *et al.* 2004) **Prefrontal cortex (↓)** (Kozlovsky *et al.* 2000)	**No evidence** (Lesort *et al.* 1999) **But Li inhibits GSK-3β** (Hong *et al.* 1997)	**Substantia nigra and upper pons (↑)** (Nagao and Hayashi, 2009)	**Associated with Tau phosphorylation** (Ferrer *et al.* 2005; Hanger *et al.* 1992)	Axon guidance, canonical Wnt receptor signalling pathway, hippocampus and superior temporal gyrus development, glycogen metabolism, NGF receptor signalling pathway
Orexin A (Hypocretin-1)	O43612 HCRT 17q21	**CSF (↓)** (Dalal *et al.* 2003) **Genetic association** (Fukunaka *et al.* 2007)	**No evidence** (Schmidt *et al.* 2010)	**Brain (↓)** (Thannickal *et al.* 2007) **CSF (↓)** (Drouot *et al.* 2003)	**Possibly** (Kang *et al.* 2009)	Activation of phospholipase C, elevation of cytosolic calcium concentration, negative regulation of DNA replicatio, negative regulation of nerve impulse, synaptic transmission
Chromogranin A (parathyroid secretory protein 1)	P10645 CHGA 14q32	**CSF (↓)** (Landen *et al.* 1999) **Brain (↓)** (Iwazaki *et al.* 2004) **Serum (↑)** (Guest *et al.* 2010) **Genetic association** (Takahashi *et al.* 2006)	**No studies**	**CSF (↓)** (O'Connor *et al.* 1993)	**Brain (↑)** (Weiler *et al.* 1990) **CSF (↓)** (Blennow *et al.* 1995)	Regulation of blood pressure, apoptosis, inhibition of dopamine release, calcium-binding, inhibition of glucose-stimulated insulin release
Neurogranin	Q92686 NRGN 11q24	**Detectable in CSF** (Davidsson *et al.* 1999) **Brain (↓)** (Broadbelt *et al.* 2006) **Genetic association** (Ruano *et al.* 2008; Stefansson *et al.* 2009)	**Animal models of mania: (↑) in amphetamine treated animals and (↓) by chronic Li treatment** (Szabo *et al.* 2009)	**No studies**	**Brain (↓)** (Davidsson and Blennow, 1998; Reddy *et al.* 2005) **CSF (↑)** (Thorsell *et al.* 2010)	Synaptic plasticity, regulates calmodulin-Ca^{2+} availability

Table 6 (continued)

Protein Name	Swissprot Accession Number Gene Symbol Gene Locus	SCZ	Bipolar Disorder	Parkinson's disease	Alzheimer's disease	Main Cell processes involved
Ras-related protein	P20336 RAB3A 19p13.2	**Detectable in CSF** (Davidsson *et al.* 1999) **Brain (↓)** (Davidsson *et al.* 1999)	**No studies**	**Possibly** (Gitler *et al.* 2008)	**Brain (↓)** (Blennow *et al.* 1996; Davidsson *et al.* 2001)	Axonogenesis, GLU secretion, mitochondrion organization, sensory perception of touch, regulation of exocytosis
Neurosecretory protein VGF	O15240 VGF 7q22.1	**CSF (↑)** (Huang *et al.* 2006a) **CSF (VGF23-62 ↑)** (Huang *et al.* 2007)	**Hippocampus and prefrontal cortex (VGF ↓)** (Thakker-Varia *et al.* 2010)	**Cortex (VGF ↓)** (Cocco *et al.* 2010)	**Cortex (VGF ↓)** (Cocco *et al.* 2010)	Insulin secretion, regulation of neuronal synaptic plasticity
Synaptotagmin	P21579 SYT1 12cen-q21	**Detectable in CSF** (Davidsson *et al.* 1999) **Brain (↑)** (Sokolov *et al.* 2000)	**No studies**	**Yes (?)** (Glavan *et al.* 2009)	**Yes (?)** (Glavan *et al.* 2009)	Synaptic transmission, detection of calcium ion, regulation of Ca-dependent exocytosis, GLU secretion
Growth-associated protein 43 (Neuromodulin)	P17677 GAP-43 3q13.1-q13.2	**Detectable in CSF** (Davidsson *et al.* 1999) **Brain (↑)** (Blennow *et al.* 1999; Sower *et al.* 1995) **Brain (↓)** (Eastwood and Harrison, 1998; Tian *et al.* 2007; Webster *et al.* 2001; Weickert *et al.* 2001)	**Brain (↓)** (Eastwood and Harrison, 2001; Tian *et al.* 2007) **CSF (↓)** (Sjogren *et al.* 2000)	**CSF (↓)** (Sjogren *et al.* 2000) **Genetic polymorphism** (Poduslo, 1993)	**CSF (↑)** (Sjogren *et al.* 2000) **Brain (↓)** (Callahan *et al.* 1994) **Genetic polymorphism** (Poduslo, 1993)	Glial derived S100B inhibits PKC phosphorylation of neuron-specific GAP-43 (Sheu *et al.* 1994), activation of PKC activity by G-Protein coupled receptor protein signalling pathway, cell differentiation, nervous system development
Synaptosomal associated protein 25	P60880 SNAP-25 20p12-p11.2	**CSF (↑)** (Thompson *et al.* 1999) **CSF (↑)** (Thompson *et al.* 2003b)	**Parietal Cortex (↑)** (Gray *et al.* 2010)	**No human studies**	**Cortex (↑)** (Dessi *et al.* 1997) **Cortex (↓)** (Greber *et al.* 1999)	Axonogenesis, calcium ion-dependent exocytosis of neurotransmitter, growth hormone secretion, regulation of insulin secretion, synaptic transmission

1.2.9. Proteomic studies in urine in schizophrenia

Urine is easily accessible and is a cell free and relatively simple tissue, something that can be both an advantage and a limitation. In addition, urine volume, composition and pH differ greatly in healthy individuals depending on diet, fluid intake, circadian rhythm, first-void versus midstream samples and exercise, making patent the need for standardised protocol for sample collection and processing prior to proteomic analysis. An international effort in setting these can be found in The Human Kidney and Urine Proteome Project website (www.hkupp.org).

Several proteins not necessarily related to renal diseases can be detected and are currently used in the clinical setting. This is the case for albumin in cardiovascular disease and β-human chorionic gonadotropin in pregnancy.

Biomarker discovery in urine involves collection of urine in sterile recipients, centrifugation, protein fractionation (Pieper *et al.* 2004) and protein separation and identification of candidate proteins using 2D-PAGE coupled with MS; or using MS/MS or SELDI (Barratt and Topham, 2007).

In SCZ, urine has been examined for potential markers of the disorder since the early 1950s (Morgan and Pilgrim, 1952) with little or no success. Urinary products identified include amino acids (McGeer *et al.* 1956), immunoglobulins , glycoproteins, the 'pink spot' (Bourdillon *et al.* 1965; Siegel and Tefft, 1971; Stabenau *et al.* 1970) and hormones (Lovegrove *et al.* 1965) and more recently, capillary electrophoresis coupled to mass spectrometry (CE-MS) has been used to examine the composition of urine and CSF (Wittke *et al.* 2005). To date, no conclusive biomarker of SCZ has been found in urine.

1.2.10. Proteomic studies in plasma in schizophrenia

For human studies, plasma is preferred to serum as changes to the proteome occur in vitro during the process of coagulation (Omenn *et al.* 2005). Sample preparation includes sample capture, transportation, storage and handling and is dependent on the aim of the experiment, techniques used and type of sample under study. Blood should be collected by trained phlebotomists using a 25G or a 23G needle to avoid mechanical cell lyses in EDTA coated tubes since EDTA is a more effective calcium chelator than citrate. Tubes are usually centrifuged as soon as possible at 3000 rpm for 8 minutes at 4°C to stabilize labile biomarkers and plasma is aliquoted and immediately frozen in liquid nitrogen or at -80°C. Repeated thaw-freeze cycles should be avoided and whenever possible, platelet depletion will be preferred to prevent the release of proteins by activated platelets. Protease inhibitors cocktails may interfere with subsequent MS experiments on the sample and their use is not routinely advised.

The analysis of the human proteome poses several challenges. The first one is its large dynamic range. In plasma, the concentration of the most abundant protein is 15 orders of magnitude more than that of least abundant proteins and, as we mentioned before only 10 proteins contribute to 90% of the whole mass of plasma proteins (Righetti *et al.* 2005).

A number of approaches to this problem have been adopted, none entirely satisfactory. One approach is to deplete the sample of the most abundant proteins such as albumin and immunoglobulins in blood for example (Pieper *et al.* 2003), but this risks indiscriminate depletion of proteins of interest as those most abundant proteins tend to be scaffold or carrier proteins, as well as rendering the sample concentration of protein too small. This low concentration will require the use of larger sample volumes which may not be possible due to limited availability of sample or to the limitations of the technique to accommodate it. Another approach is to fractionate the sample and to look at intracellular, extracellular, nuclear or mitochondrial content specifically. This works well also but with the drawback that the analytical challenge is multiplied by the number of fractions drawn and that fractioning may imply a loss of sample.

Recent evidence suggests that the study of blood cells such as circulating lymphocytes might offer a suitable alternative to studying brain metabolism in neuropsychiatric disorders such as depression, stress, Alzheimer's disease (AD) and SCZ (Gladkevich *et al.* 2004) . 2DGE-MS was successfully used by Prabakaran and colleagues on liver and red blood cells to identify protein alterations related to oxidative stress in SCZ (Prabakaran *et al.* 2007) and a series of studies have identified gene expression changes in circulating blood cells that differentiate SCZ from controls (Middleton *et al.* 2005).

Thus, a bibliographic search was conducted using the terms 'plasma', 'SCZ' and 'protein' and 1194 papers were obtained. The earliest papers were by Man et al., published in 1947 and describing variations in plasma aminoacids and nitrogen during exacerbations of the disorder and periods of recovery (Man *et al.* 1947b) as well as following prefrontal lobotomy (Man *et al.* 1947a).

The search was narrowed down by using the following terms: 'blood', 'electrophoresis', 'SCZ' and 'protein' and 139 papers were retrieved and those form 1975, the year in which 2DPAGE was established as a proteomic technique were studied (88 in total). Papers reporting discovery in cell lines (lymphoblastoid, red blood cells and platelets) were also included.

Proteins identified as upregulated in serum according to one study only are:

1. Alpha-2 globulin and beta-globulin (Varma and Hoshino, 1980).

2. IgG heavy chain (50 kD prolactin binding protein) (Walker *et al.* 1992).
3. 60 kDa Human Heat-Shock Protein (HSP) that is the P1 mitochondrial protein (Kilidireas *et al.* 1992).
4. Retroviral protein (HERV)(ERV9) (Huang *et al.* 2006b).
5. Chromogranin A (parathyroid secretory protein 1) (CHGA) was also upregulated in serum (Guest *et al.* 2010).
6. Neuron-specific enolase (gamma enolase, enolase 2) was found to be increased in serum of patients with SCZ (Medina-Hernandez *et al.* 2007) and it was found to be decreased in the plasma of people with bipolar disorder in another study (Machado-Vieira *et al.* 2007).

The following have been found upregulated in plasma in one study:

1. Reelin was found in plasma of patients with SCZ, depression and bipolar disorder (Fatemi *et al.* 2001).
2. α1-antitrypsin, serum amyloid P-component, α1-microglobulin, antithrombin III and vitamin D binding protein (Wan *et al.* 2007).

Whereas the following were found downregulated in the plasma of patients with SCZ:

1. Haptoglobin beta chain, α1-antitrypsin, complement factor B precursor, apolipoprotein A-IV, and apolipoprotein A-I (Yang *et al.* 2006).
2. Apolipoprotein E appeared to be downregulated in the plasma of both in SCZ and bipolar disorder patients (Dean *et al.* 2008).

The following appeared to be differentially expressed in cellular fractions:

1. FA/GSK-3 α (activity and levels in lymphocytes were downregulated according to a study by Yang and colleagues in 1995 (Yang *et al.* 1995).
2. H2B (histone), an acid protein from the plasma membrane of lymphocytes was found to be downregulated (Sourlingas *et al.* 2003).
3. In a study of red blood cells, selenium binding protein (SBP1) and glutathione-S-transferase (GSTA3) were found to be upregulated; whereas, serum albumin (ALB), actin (ACTB), erythoid α spectrin (SPTA1), apolipoprotein A1 (APOA1), thioredoxin peroxidase (peroxiredoxin 5) (PRDX5) and heat-shock 70kDa protein-like 1 (HSPA1L) appeared to be downregulated (Prabakaran *et al.* 2007).
4. The Fyn protein was downregulated in platelets in another study (Hattori *et al.* 2009).

Another proteins detected in plasma and for which differential expression has not been found are creatin kinase BB isoenzyme (Lerner and Friedhoff, 1980); Platelet MAO (Yu *et al.* 1982) or erythrocyte membrane proteins (Fritze *et al.* 1988).

Proteins for which two or more studies have been conducted and the changes in their expression are concordant are shown in Table 7 and a summary of those proteins for which expression has been found to be concordantly differentially expressed in at least two different tissues (plasma, CSF, brain or white matter) is shown in Table 8.

Most proteins identified using proteomic techniques so far have an average molecular weight of 39964.2558 daltons (Da), ranging from NRG (7618 Da) to ACE (149751 Da).

1.2.11. Limitations of blood-based proteomic biomarkers

Plasma is collected almost effortlessly and in large quantities from human populations and constitutes one of the most informative samples for assessing the state of health of the individual. The study of human plasma using proteomic techniques has the potential of yielding information about all the differentiated sub-proteomes of the body, as plasma contains detectable quantities of combinations of proteins that are tissue-specific and potentially informative of disease states. The plasma proteome has been examined using 2DPAGE coupled with LC/MS/MS for the past 30 years resulting in the identification and characterization of hundreds of plasma proteins, facilitating the creation of the Plasma Proteome Project (PPP) by the Human Proteome Organisation (HUPO) (http://www.hupo.org/research/hppp/). The main aim of the PPP is to stimulate large-scale analysis of human plasma and serum and open sharing of results so that a foundation for biology- and disease-driven components can be established. To date, over 1929 protein sequences at a false discovery rate of 1% have been identified and annotated (Farrah *et al.* 2011).

The first challenge posed by plasma is that it has a large dynamic range, exceeding 10^{10} (from albumin at around 45 mg/mL to some cytokine at 1-10 pg/mL), whereas proteomic techniques have a narrower level of detection 10^4 to 10^6 and candidate biomarkers are likely to be low abundant. Highly abundant proteins (those found at concentrations higher than 1µg/mL are involved in proteolysis, binding, transport, coagulation and immune response and are mostly synthesised by the liver and the intestines.

Plasma proteins are classified as (1) “classic” proteins whose function depends on their presence in plasma, and (2) transient, secreted proteins which utilise plasma for transportation or for dilution and filtered removal effects (Anderson and Anderson, 2002; Farrah *et al.* 2011).

“Classic” proteins account for approximately 13% of the total plasma protein content and are well characterized and used for diagnostic purposes (i.e. C-reactive protein, serum amyloid, myogoglobin and ferritin amongst many others). Classic proteins are mostly secreted by the liver and intestines and perform their function (proteolysis, inhibition, binding, transport, coagulation and immune response chiefly) in the blood, where they are found at a concentration > 1 µg/mL (Jacobs *et al.* 2005).

The second class of proteins contains the subgroup of secreted proteins (cytokines, receptor ligands and hormones) that utilise plasma as a vehicle for transportation and mediation of cellular responses, and a subgroup of intracellular proteins believed to originate from cellular damage or leakage. This less characterised group of proteins with low total concentrations ranging from µg/mL to pg/mL is probably the most interesting one for potential biomarker studies and the more challenging to work with.

Direct proteomic plasma analysis, particularly for this second group of proteins, is extremely challenging because of the many factors surrounding intrinsic plasma proteins variations, sample collection, processing and storage methods, prior to protein analysis, detection and identification. For that reason, it is crucial to attempt optimisation of the process by preparing the sample prior to analysis, enriching the content of the proteins of interest to improve their detection and by improving current technology to allow better detection and more accurate identification of analytes of interest.

The target of most proteomic studies is to identify candidate biomarkers to differentiate between diseased and normal states of health, or to improve prediction, detection, and prognosis or treatment response. These disease-related biomarkers would only appear in plasma during abnormal physiological states as a result of altered cellular pathways in the tissue of interest. Therefore, these targets will only be present in extremely low quantities in plasma, testing the technical range of detection of proteomic technological platforms. However, it is unlikely that there will be any single technology capable of covering the full dynamic range, at least for the foreseeable future

A second challenge of blood-based biomarker studies is posed by the complexity of proteins. Proteins have different isoforms, they are secreted and metabolised in different ways, they may bind to other proteins to form stable complexes or to achieve a particular function, they bind to other molecules and substrates such as lipids, they have active and inactive forms, and a potentially large number of post-translational modifications.

This level of complexity has made necessary the development of increasingly sophisticated bioinformatics tools and mass spectrometry techniques. Sample separation

techniques, which can be either gel (2D PAGE with silver or fluorescent staining) or non-gel (LC) based, are necessary as a first step to reduce the complexity of the sample, and a combination of those is likely to be needed as a first step in discovery approaches. This initial step inherently results in a reduced range of analytes being examined, leaving some potentially useful biomarkers outside the detection range of subsequent analysis.

Different gel-staining techniques have their own limitations and although differential gel electrophoresis (DIGE) allows for direct case-control comparison within the same gel, silver staining is considered to be more sensitive. Mass spectrometry-based proteomic techniques (such as multiple reaction monitoring, LC/MS/MS, isobaric tagging, MALDI and SELDI) are also varied and their use will depend largely on the experimental design of the study.

A 'candidate protein approach' by which antibodies are used detect specific analytes can also be helpful in hypothesis-driven experiments. The obvious limitation is the availability of a specific and sensitive commercial antibody but recent advances such as the Luminex xMAP® system has allowed multiplex assay of up to 100 proteins relatively rapidly and reliably. Another limitation of this approach is that its outcome either confirms or refutes the original hypothesis but does not generate new ones which, in a discipline as relatively new to proteomics such as psychiatry, may narrow discovery and prevent new hypothesis generation.

Once a protein or a group of proteins have been found to be differentially expressed in disease-related states, it is necessary to validate and to replicate the finding. Validation is performed by using a different technique in the discovery sample, this time using a hypothesis-led approach. The main challenge in validation is that different, albeit overlapping proteome are canvassed as a result of the differences in the detection ranges of the techniques used. Replication is usually achieved by using an independent sample to the one used in the discovery phase. The limitations in human based studies are obvious: no two samples are identical, and since protein expression is altered by environmental factors as much as by genetic ones, the chances of validating the target are severely reduced. The advantage is the high reliability of the finding if validation occurs.

A final stage of biomarker discovery is asserting whether the potential candidate can be used in clinical trials as an indicator of treatment response, or have enough negative and predictive power to serve as a diagnostic marker, or whether it makes for a reliable surrogate marker of the disorder (Thambisetty and Lovestone, 2010).

Table 7. Protein candidates from proteomic studies in human plasma in SCZ.

Protein Name	Accession No. Gene Symbol Gene Locus	Plasma in SCZ direction of regulation and replication studies	Bipolar Disorder	Parkinson's Disease	Alzheimer's' Disease	Main processes involved
Brain derived neurotrophic factor	P23560 BDNF 11p13	**Serum (↑)** (Jockers-Scherubl *et al.* 2004) **Plasma (↓)** (Palomino *et al.* 2006; Tan *et al.* 2005)	**Serum (↓)** (Cunha *et al.* 2006) **Plasma (↓)** (Palomino *et al.* 2006)			Nervous system development
Nerve Growth Factor	P01138 NGF 1p13.1	**Plasma (↓)** (Kale *et al.* 2009) **Serum (↓)** (Xiong *et al.* 2010)	**Plasma (↓)** (Barbosa *et al.* 2010)	**Serum (↓)** (Lorigados *et al.* 2002)		Apoptosis, inflamatory response, activation of phospholipase C, axogenesis, response to drug, electrical stimulus and to glucocorticoid stimulus
Transthyretin (Prealbumin)	P02766 TTR 18q12.1	**Plasma (↓)** (Wan *et al.* 2006; Yang *et al.* 2006)	**Genetic association** (Mors *et al.* 1997) **Li down regulated TTR mRNA** (Pulford *et al.* 2006)		**Serum (↓)** (Elovaara *et al.* 1986)	Intra/extra cellular transport, hormone binding
Interleukin-6	P05231 IL6 7p21	**Serum (↑)** (Maes *et al.* 1995a; Naudin *et al.* 1996) **Serum IL6R (↑)** (Muller *et al.* 1997)	**Plasma (↑)** (Maes *et al.* 1995a)		**Blood (↑)** (Swardfager *et al.* 2010)	Acute phase immune response, cell redox homeostasis, negative regulation of apoptosis
Interleukin-2	P60568 IL2 4q26-q27	**Serum (↑)** (Ganguli and Rabin, 1989) **Plasma (↑)** (Maes *et al.* 1995b)	**Plasma (↑)** (Maes *et al.* 1995a)	**Serum (↑)** (Brodacki *et al.* 2008) **PBMC (↓)** (Bessler *et al.* 1999)	**PBMC (↓)** (Beloosesky *et al.* 2002) **MNC (↑)** (Huberman *et al.* 1994)	Negative regulation of: apoptosis, heart contraction, inflammatory response, lymphocyte proliferation, protein phosphorylation. Positive regulation of: cell growth and proliferation, immunoglobulin secretion, phosphorylation and tissue remodelling and elevation of cytosolic calcium ion concentration
Neural cell adhesion molecule 1	P13591 NCAM1 11q23.1	**Serum (↑)** (Lyons *et al.* 1988) **Serum alterations** (Tanaka *et al.* 2007)			**Serum (↑)** (Todaro *et al.* 2004)	Axon guidance, cell adhesion, cytokine-mediated signalling pathway, positive regulation of calcium-mediated signalling
Haptoglobin α2 chain	P00738 HP 16q22.1	**Plasma(↑)** (Maes *et al.* 1997; Wan *et al.* 2007; Yang *et al.* 2006)	**Plasma(↑)** (Maes *et al.* 1997)			Cellular iron homeostasis, immune response. positive regulation of cell death, proteolysis, response to hydrogen peroxide
Cholecystokinin	CKK P06307 3p22-p21.3	**Peripheral blood mononuclear cells (↑)** (Mauri *et al.* 1998; Panza *et al.* 1992)				Activation of PKC activity, axonogenesis, neuron migration. Positive regulation of apoptosis, cell proliferation and mitochondrial depolarization. Regulation of sensory perception of pain. Release of cytochrome c from mitochondria

Table 8. Protein candidates from SCZ Proteomic studies ordered by gene locus.

Protein Name	Symbol	Gene Locus	Accession Number	Molecular Weight	Plasma	CSF	Brain	White matter
Internexin Neuronal Intermediate Filament Protein, Alpha	INA	10q24.33	Q16352	55391			Y	
Phosphoglycerate mutase 1	PGAM1	10q25.3	P18669	28804			Y	
Brain derived neurotrophic factor	BDNF	11p13	P23560	27818	Y	Y		
Neural cell adhesion molecule 1	NCAM1	11q23.1	P13591	94574	Y	Y		
Neurogranin	NRGN	11q24	Q92686	7618		Y		
Synaptotagmin	SYT1	12cen-q21	P21579	47573		Y		
l-Lactate Dehydrogenase Chain *B*	LDHB	12p12.2-p12.1	P07195	36638				Y
Enolase 2 (gamma, neuronal)	ENO2	12p13	P09104	47269		Y	Y	
Neurotensin	NTS	12q21	P30990	19795		Y		
Creatine Kinase, Brain	CKB	14q32	P12277	42644			Y	
Chromogranin A (parathyroid secretory protein 1)	CHGA	14q32	P10645	50688		Y		
Haptoglobin α2 chain	HP	16q22.1	P00738	45205	Y			
Aldolase C, Fructose-Bisphosphate	ALDOC	17cen-q12	P09972	39456			Y	
Orexin A, (Hypocretin-1)	HCRT	17q21	O43612	13363		Y		
Glial Fibrillary Acidic Protein	GFAP	17q21	P14136	49880			Y	
N-Ethylmaleimide-Sensitive Factor	NSF	17q21	P46459	82594			Y	
Angiotensin I-Converting Enzyme	ACE	17q23.3	P12821	149751		Y		
Transthyretin (Prealbumin)	TTR	18q12.1	P02766	15887	Y	Y		
Peroxiredoxin 2	PRDX2	19p13.2	P32119	21892			Y	Y
Ras-related protein	RAB3A	19p13.2	P20336	24984		Y		
Nerve Growth Factor	NGF	1p13.1	P01138	26959	Y	Y		
Triosephosphate Isomerase 1	TPI1	1p31.1	P60174	26943			Y	
Stathmin 1	STMN1	1p36.1	P16949	17303				Y
Parkinson Disease 7	PARK7	1p36.33	Q99497	19891				Y
Guanine Nucleotide Binding Protein, Beta Polypeptide 1	GNB1	1p36.33	P62873	37377			Y	
Enolase 1 (α)	ENO1	1p36.3–p36.2	P06733	47169			Y	
GLU-Ammonia Ligase	GLUL	1q31	P15104	42064			Y	
Synaptosomal associated protein 25	SNAP-25	20p12-p11.2	P60880	23315		Y		
S100 calcium binding protein B	S100B	21q22.3	P04271	10713		Y		
Septin 3	SEPT03	22q13.2	Q9UH03	40704			Y	
Cholecystokinin	CKK	3p22-p21.3	P06307	12669	Y	Y		
Growth-associated protein 43, (Neuromodulin)	GAP-43	3q13.1-q13.2	P17677	24803		Y		
Glycogen synthase kinase 3 beta	GSK3B	3q13.3	Q6FI27	46744		Y		
Thyrotropin-Releasing Hormone	TRH	3q13.3-q21	P20396	27404		Y		
Ubiquitin Carboxyl-Terminal Esterase L1	UCH-L1	4p14	P09936	24824			Y	
Interleukin-2	IL2	4q26-q27	P60568	17628	Y	Y		
Interleukin-6	IL6	7p21	P05231	23718	Y	Y		
Neurosecretory protein VGF	VGF	7q22.1	O15240	67258		Y		
Neurofilament, Light Polypeptide	NEFL	8p21	P07196	61517				Y
Dihydropyrimidinase-Like 2	DPYSL2	8p22-p21	Q16555	62294			Y	Y
Inositol-Monophosphatase 1	IMPA1	8q21	P29218	30189				Y
14-3-3 zeta	YWHAZ	8q23.1	P63104	27745				Y
Dynamin 1	DNM1	9q34	Q05193	97408			Y	

Proteins found to be present in at least two human tissues are highlighted.

1.3. Summary

SCZ is a clinical entity that arises as a result of abnormal brain functioning. The phenotypes that clinicians identify with this diagnostic category are likely to be the result of complex interactions between diverse, albeit probably convergent, molecular pathways and environmental influxes. Assuming that such a pathological process exists and can be detected, is unlikely to mirror the diagnostic categories or the detailed subdivisions of diagnosis in ICD-10 or DSM-IV. The concept of SCZ is mostly helpful for conveying clinical prognosis but it is less so for helping explaining those symptoms in terms of the cellular or molecular processes that cause them.

At the core of this disorder (or group of disorders) there may be an undetermined number of genes, spread throughout the genome and coding for proteins with structural or functional roles, which additive effect disrupt brain development and function largely by altering the delicate interplay existing at the core of molecular and signalling events leading to aberrant cytostructure and rendering neuronal transmission largely inefficient. Convergent evidence for linkage studies and proteomic studies in SCZ that have been conducted mainly using a hypothesis-free approaches are already identifying putative altered molecular pathways in processes such as neuronal migration and differentiation, axonogenesis, apoptosis, inflammatory response, mitochondrial function, calcium metabolism, glutamate release and NT uptake; and cellular response to glucocorticoids, insulin, hypoxia, cocaine, and amphetamines.

Environmental factors such as in utero adverse events, peri-natal hypoxia, childhood trauma, cannabis use and adverse social events appear to modulate and in most cases precipitate the onset of frank psychotic symptomatology resulting in the diagnosis of SCZ.

Once established, psychotic symptomatology is linked to wide spread abnormal neurotransmission and hence, alterations in dopamine signalling, along with abhorrent glutamatergic, gabaergic, cholinergic, noradrenergic, endocannabinoid and serotonergic transmission are consistently found in people with schizophrenia. In addition, post-mortem and neuroimaging studies report structural anomalies such as reduced overall brain weight, alterations of hemispheral symmetry, enlarged ventricles, thinning of grey matter, abnormal neuronal migration and apoptosis and alterations in synaptic connection, prunning and dendrite function; and functional alterations such as abnormal cerebral blood flow, metabolic rate for glucose, oxygen metabolism, receptor density and neurotransmitter release. These alterations appear to precede the onset of symptoms and, in certain patients, their progression leads to an end-stage state of marked cognitive deficit not too dissimilar from the

clinical picture seen in dementing neurodegenerative disorders, which suggest that a core neurodevelopmental process is furthered aggravated by a neurodegeneration component.

Systemically, people with schizophrenia appear to have altered immunological responses, with blunted levels of type-1 activating cytokines and raised levels of type-2 (anti-inflammatory) cytokines and a shift from cellular to humoral immune response which is also present centrally in glial cells. In addition, schizophrenia patients display deficiencies in energetic metabolism and mitochondrial function which have been detected peripherally and centrally.

This suggests that the proteomic profile of peripheral tissues may show subtle differences in SCZ. These differences could be attributable to changes on the brain but also may suggest that SCZ is part of a heterogeneous group of 'signalling disorders' in which the brain alteration responsible for the symptoms accompanies a peripheral change that might be detectable.

To date, relatively few attempts have been made to seek biomarkers in SCZ using so-called unbiased or data-driven technologies such as proteomics. However, there are indications that such an approach is not only possible but also productive.

Molecular biomarker research can be done using genomic, transcriptomic, metabolomic and proteomic techniques. Genomic studies have identified mostly trait markers, that is whether a genetic predisposition exist that confers a higher risk to developing psychosis, although state markers are currently being investigated using epigenetic approaches. Transcriptomic concerns the study of RNA transcripts and they it has been successfully used in examining post-mortem brain tissue and peripheral blood samples. Metabolomic research examines analytes such as lipids, aminoacids, nucleotides and sugars which are downstream from the genome, transcriptome and proteome and closely reflects cell activity.

Proteomic techniques have already been applied in SCZ research using post-mortem brain tissue, CSF, lymphocytes, serum and plasma. The following proteins have been found in at least two of the former tissues: brain derived neurotrophic factor (BDNF); neural cell adhesion molecule 1 (NCAM1); enolase 2 (gamma, neuronal) (ENO2); transthyretin (Prealbumin) (TTR); peroxiredoxin 2 (PRDX2); nerve growth factor (NGF); cholecystokinin (CKK); interleukin-2 (IL2); interleukin-6 (IL6) and dihydropyrimidinase-like 2 (DPYSL2).

Nonetheless, the extent to which these alterations are consequence of the disorder(s) that lay at the core of the symptoms, or are the reflections of environmental modulators, the treatment the subjects were receiving or the circumstances surrounding sample collection,

processing and storage, which could be of particular relevance in post-mortem studies, for instance, is unknown.

In this thesis, a consistent proteomic approach following established standard operational protocols for collection, preparation and storage of the samples will be used to (1) study a molecular model of the disorder using a DISC1 knock-down human neuronal cell line; (2) the effects of medication on glial cells and (3) in the brain and plasma of F344 rats; and (4) to canvass the plasma of people with psychosis for surrogate markers of the disease.

The discovery phase was performed in neuronal and glial cell cultures, animal brain tissue, and animal and human plasma using two dimensional gel electrophoresis for protein separation in complex samples coupled with mass spectrometry for protein identification. The validation phase for potential biomarkers that could help identify underlying molecular changes responsible for psychotic symptoms; serve as diagnostic markers, or markers for severity of symptoms, or to refine the clinical phenotype; to inform drug discovery and serve as markers of toxicity or of biological action of antipsychotic treatment was done, when applicable, with immunoblotting (Western blot and ELISA).

CHAPTER 2: Methods

2.1. Materials for 2DPAGE

Material	Supplier
Acrylamide PAGE, PlusOne, 40%	GE Biosciences
Acetic acid	BDH
Agarose, low melting point	Sigma
Ammonium hydroxide	Sigma
Ammonium persulphate	Sigma
Bromophenol blue	Sigma
Citric acid	Sigma
CHAPS	BDH
DeStreak rehydration solution	GE Biosciences
Dithiothreitol	Sigma
Ethanol	VWR
Formaldehyde solution	Sigma
Glutaraldehyde solution	Sigma
Glycerol, for molecular biology, min.99%	Sigma
Glycine	Sigma
Hydrochloric acid	Sigma
Immobiline Drystrip gels, pH 3-11 NL, 18cm	GE Biosciences
IPG Buffer, 7-11 NL	GE Biosciences
IPGphor Strip Holder Cleaning Solution	GE Biosciences
Iodoacetamide, SigmaUltra	Sigma
Isopropanol	Sigma
Mineral Oil	Sigma
Napthelene sulphonic acid	Sigma
Piperazine diacrylamide (PDA), 1,4-Bis(acryloyl)piperazine, crosslinker for electrophoresis	Biorad
Resolving Buffer 4x (Protogel)	National Diagnostics
Silver nitrate	VWR
Sodium acetate, SigmaUltra, min. 99%	Sigma
Sodium dodecyl sulphate (SDS), for molecular biology, ~99%	Sigma
SDS, 20 % solution	Sigma
Sodium hydroxide	Sigma
Sodium thiosulphate pentahydrate, SigmaUltra, min. 99.5%	Sigma
Strip Holder Cleaning Solution	GE Biosciences
TEMED	Sigma
Thiourea	Sigma
TMD-8,	Sigma
Trizma base	Sigma
Trizma base, SigmaUltra, min. 99%	Sigma
Ultrapure Acrylamide 30% (ProtoGel)	National Diagnostics
Urea, SigmaUltra	Sigma

Chemicals were purchased from Sigma-Aldrich Chemical Company, VWR international and GE Biosciences, unless otherwise stated. Ultra pure water (ddH2O) from an Elga Maxima water purification system was to make up all solutions.

2.2. A-Z of solutions

Acrylamide Solution (12 gels)	Acrylamide/PDA solution (10%)(250 ml) Resolving Buffer solution (1.5M Tris-HCL pH8.8) (1x) (187.5 ml) Ultra pure water (300 ml) Sodium thiosulphate (0.08%) (3 ml) SDS (0.1%) (3.75 ml) Ammonium persulphate (0.067%) (5 ml) TEMED (1/1500) (500 µl)
Agarose sealing solution	SDS electrophoresis buffer containing: 0.5% agarose 0.002% bromophenol blue
Bradford solution	1:5
Developing solution	0.005% citric acid 0.15% formaldehyde
IPG rehydration buffer	3 ml DeStreak Rehydration Solution 15 µl IPG Buffer (pH3-11 NL)
PBS-Tween (PBS-T)	5 tablets PBS in 1l dd H_2O 0.2% (v/v) Tween 20
PBS-T-milk	PBS-T 5% (w/v) dry non-fat milk (Marvel)
Ponceau Solution	4% (v/v) glacial acetic acid 0.7% (w/v) Ponceau S Ponceau 0.2 g Trichloroacetic acid (TCA) 5 g dd H_2O up to 100 ml
Resolving gel	125 mM Tris-HCl, pH 8.8 0.1% (w/v) SDS 10% (w/v) stock acrylamide 0.10% (v/v) TEMED 10 µl/ml of 10% (w/v) ammonium
SDS electrophoresis buffer	25 mM Tris-base 192 mM glycine 0.1% (w/v) SDS
SDS equilibration buffer	50 mM Tris-HCl pH 8.8 6 M urea 30% (v/v) glycerol 2% SDS 0.002% bromophenol blue
Sealing solution	Agarose (0.5%) SDS electrophoresis buffer
Silver stain solution	0.013% (w/v) silver nitrate 10 mM HCl 0.013% (v/v) ammonium hydroxide
Standard lysis buffer (50ml)	9.5M Urea 2% CHAPS 0.8% Pharmalyte pH 3-10 (optional) 1% DTT
Strong fix	40 % ethanol, 10 % glacial acetic acid
Stop solution	5% (w/v) Tris-base 2% (v/v) glacial acetic acid

Stacking gel	125 mM Tris-HCl, pH6.8 0.1% (w/v) SDS 3.75% (w/v) stock acrylamide 0.15% (v/v) TEMED 6 μl/ml of 10% (w/v) ammonium persulphate
Tris buffered saline (TBS)	25 mM Tris pH 8.0 140 mM sodium chloride 5 mM potassium chloride
Transfer buffer	25 mM Tris, pH 8.3 182 mM glycine 20% (v/v) methanol
TBS-TB	TBS-T 5% (w/v) BSA
Weak fix	5 % ethanol 5 % glacial acetic acid
Western blot Acrylamide Solution	Ultrapure Acrylamide 30% (Protogel) 10 ml Resolving Buffer 4x (Protogel) 7.5 ml ddH2O 12.2 ml APS (10%) 300 μl (Add immediately before pouring) TEMED 30 μl (Add immediately before pouring)
Western blot Stacking Gel	Ultrapure Acrylamide 30% (Protogel) 2 ml Resolving Buffer 4x (Protogel) 3.75 ml ddH2O 9.2 ml APS (10%) 70 μl (Add immediately before pouring) TEMED 15 μl (Add immediately before pouring)
Wet Transfer Buffer	25 mM TRIS (7.6 g) 192 mM Glycine (36 g) 20% v/v Methanol (500 ml) 0.025% w/v SDS (3.13 ml of 20% stock) ddH2O to a total volume of 2.5 l

2.3. Sample preparation: collection, processing and storage

2.3.1. Neuronal cell culture and treatment (Chapter 3)

a. Culturing of CTXOE03 cells

The CTXOE03 neural progenitor cell line is a karyotypically normal, conditionally immortalized line derived from first trimester human cortical neuroepithelium (Pollock *et al.* 2006) and obtained from ReNeuron Ltd. Cells were grown on laminin at 37°C (5% CO2) with 10ml DMEM F12 media per T75 flask, supplemented with human albumin serum (0.75ml, 20%), transferrin (1ml, 50mg/ml), putrescine DiHCl (8.1 mg/ml), insulin (0.25 ml, 10mg/ml), progesterone (1ml, 20µg/ml), L-glutamine (5ml, 200 nM), sodium selenite (1 ml, 20 µg/ml), bFGF (0.5 ml, 10 µg/ml), EGF (1 ml, 10 µg/ml), and 100nM 4-OHT.

b. RNAi Transfection of CTXOE03 cells

RNAi transfection was carried out using the NTER system (Sigma). Transfection of cortical cells was carried out twice: 24 hours after seeding and again 72 hours later. There were 4 RNAi conditions - 'Control RNAi', 'RNAi1', 'RNAi2' and 'No RNAi' – with six T75 flasks in each condition (designated A-F). Cells in the 'Control RNAi' condition were transfected on each occasion with 10nM Stealth Negative Control Duplex (Medium GC), which is non-homologous for any known vertebrate transcript. Cells in the 'RNAi1' condition were transfected on each occasion with siRNA specific to DISC1 (DISC1 HSS120484 CCCUCAACUUGUCACUUAAAGAAAU; exon 7). Cells in the 'RNAi2' condition were transfected on each occasion with a second siRNA specific to DISC1 sequence (DISC1 HSS120484 CCCUGAGGAAGAAAGUUAACGAUAU; exon 8). Cells in the 'No RNAi' condition were not treated with any siRNA. As an index of transfection efficiency, a separate T75 flask of CTXOE03 cells was transfected in the same manner as above but treated with BLOCK-iT Alexa Fluor Red Flourescent Oligo, in place of the respective siRNAs used for the other conditions. Cells were harvested six days after initial siRNA transfection. Cells were removed from flasks using versene and pelleted by centrifugation at 900RPM for 5 min, with two PBS washes. From each T75 flask, half of the cells were pelleted for subsequent RNA extraction and half for protein extraction. RNA extraction was performed using Tri Reagent (Ambion).

c. cDNA synthesis and qPCR

cDNA synthesis was carried out using Superscript III (Invitrogen), following DNase treatment of extracted total RNA. qPCR to assess DISC1 RNA knockdown was carried out using SYBRGreen measurement. DISC1 primer sequence: forward primer = tgaagtcacactggcgtttc; reverse primer = tgcttttgctttgtttcctg. POLR3C (housekeeper) primer sequence: forward primer= gacgaaaccatcttcctgct; reverse primer = catccaggcacctcctttat. A Relative Standard Curve was generated and the dilutions given arbitrary values based on their relative concentrations. H2O was used as a blank. Six cDNA samples in each of three RNAi conditions (RNAi1, RNAi2, and Control RNAi) were assayed in duplicate.

2.3.2. Rat brain tissue and plasma (Chapter 4)

Thirty-six adult male inbred F344 rats, weighing 225-250g on arrival, were housed in cages with a standard 12-hour light/dark cycle at 22^{o}C. Access to water and food consisting on a standard diet for rodents were provided *ad libitum*. Weight gain was monitored for the 14 days of treatment duration.

The environment including temperature, humidity, ventilation, lighting and noise was maintained according to standards set by the Home Office in the Code of Practice for the housing of animals used in scientific procedures (21/03/2005).

Animals subjected to the same treatment (haloperidol, citalopram or control) were housed together in cages of four individuals each which resulted in three cages per treatment arm (each arm consisted of 12 animals). Animals were sacrificed in three consecutive days to minimise batch effects as follows: four 'test' animals and four controls were sacrificed on day one; 12 haloperidol-treated animals and 4 control animals on day two; and 12 citalopram-treated animals and four controls on day three.

The subcutaneous implantation of chronic continuous dosing Alzet® osmotic mini-pumps to deliver the selected treatment was performed by Dr Natesan according to the procedure described by Samaha et al (Samaha *et al.* 2007). In brief, animals were anesthetised and minipumps containing vehicle (VEH; 0.5% glacial acetic acid/H_2O solution), haloperidol, or citalopram were implanted thorough a 1.5-cm-wide incision in the animal's lower back and inserted between the scapulae. The dose of the antipsychotic was chosen based on D2/3 receptor occupancy level determined in Sprague Dawley rats to ensure that clinically appropriate levels were achieved (Natesan *et al.* 2008) while the dose of citalopram was adjusted according to the equivalent human dose as current practice in animal studies (Hesketh *et al.* 2007). Haloperidol was administered to the equivalent of 0.5 mg/kg/day and

citalopram to 10 mg/Kg/day. Both agents were dissolved in the common solvent system which served both as vehicle and to treat the control group.

Animal manipulations, sacrifice and relevant tissue dissections, excisions and extractions were performed by Dr Natesan. Animals were sacrificed by decapitation on day 14 of treatment. Brains were removed and the striatum area was dissected, washed on 0.1% DEPC (diethyl pyrocarbonate), snap frozen by placing them in isopentane (methylbutane, C5H12) over dry-ice and subsequently stored at -80°C. Brain lysates were obtained using a standard lysis buffer, in which 30 g of urea were dissolved in ddH_2O, making a total volume of 50 ml. 0.5 g amberlite was added and the solution was stirred for 10 minutes before being filtered. Following filtration, 1 g CHAPS, 0.5 g DTT and protease inhibitors were added to 48 ml of the solution and it was then aliquoted into 1 ml and stored at -80°C until use.

Brain tissue was homogenized at approximately 100mg/ml in lysis buffer using a manual tissue grinder. The tissue was subjected to grinding for 2 to 3 minutes with an up and down twist motion until all tissue was homogenised. The homogenised sample was then transferred to a clean microtube and spun at 15000 rpm for 20 minutes. The resulting precipitated tissue pellet was discarded and the supernatant was retained as the total lysate. A Bradford assay (Bradford, 1976) was subsequently performed to calculate the protein concentration for each sample.

Truncal blood from sacrificed animals was collected in EDTA coated tubes immediately after decapitation using single-use sterile funnels and EDTA tubes. The tubes were placed at 4^{o}C for one hour and then spun at 3000 g for 8 minutes at 4^{o}C.

Plasma was aliquoted in 0.5 ml Eppendorf tubes and stored at -80^{o}C until needed for proteomic analyses.

2.3.3. Human plasma (Chapter 5)

Samples were collected using a 23 gauge butterfly needle and vacutainer needle holder following standard venipuncture in the cubital region, in the area of anastomosis between the radial and the ulnar veins or in the braquial vein, slightly proximal to this area. To avoid coagulation of the sample, a 9ml K3EDTA tube (Greiner Bio-One, Cat # 455036X - EDTA tube, lavender lid) was used. The sample was immediately kept at +4°C until being processed, with the time between collection and processing of the sample never exceeding two hours. The tube was labelled with the subject barcode with no phenotypic information given at this stage. The sample was then centrifuged at 3000 rcf (xg), 8 minutes at +4°C and plasma was aliquoted in 0.5 ml Eppendorf tubes and immediately frozen at -80°C.

2.4. Determination of protein concentration (Bradford assay protocol)

Protein concentrations of plasma samples were determined according to the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard. Bradford solution was made 1:5. Samples (2 μl) were mixed with Bradford reagent (1 ml) and the absorbance was read after 20 minutes at 595 nm.

Three tubes / vials per sample were labelled and placed in a rack. A 1/100 dilution of each sample was made (3 per sample). BSA standards were made up. Bradford reagent was diluted 1:5 with ddH_2O. Diluted Bradford reagent was pipette (1 ml) into each optic cuvette using an automatic pipette. Into each cuvette, 10 μl standard or sample was added (1 x reference, 3 x each standard, 3 x each sample).

Cuvettes were then vortexed to homogenise the contents and incubated for 10 minutes to 1 hour. Samples were read using the spectrophotometer @ 595 nm.

2.5. Two dimensional polyacrylamide gel electrophoresis (2DPAGE)

2.5.1. First dimension

Samples were defrosted on ice to minimise protein degradation and then mixed (vortex) and centrifuged (1 min, 13000 rpm, RT). The volume of plasma determined to contain 50 μg of protein (approximately 1 μl) was dissolved in IPG rehydration buffer (3 ml DeStreak Rehydration Solution containing 15 μl IPG Buffer (pH3-11 NL)) as follows: for running 18cm Immobiline DryStrips, 350 μl total volume was used per strip. The sample comprised no more than 100 μl of the total volume and hence, Eppendorfs containing 350 μl – $X_{sample\ volume}$ of IPG rehydration buffer + $X_{sample\ volume}$ were prepared and left at room temperature for 30 min to allow protein denaturation, followed by a 5 min spin at 13000 rpm.

Immobiline pH gradient (IPG) strips of 18 cm length (pH 3-11) were used to immobilise individual proteins according to their isoelectric point. The strips were rehydrated and focused using the IPGphor™ (Amersham Amerham biotech, UK) platform. For the rehydration phase, 350 μl of the plasma/rehydration solution were placed in each individual strip holder and subjected to a current of 50 μA per strip for 12 h at RT. The subsequent IPG electrofocusing of the rehydrated strips was carried out overnight in the same platform using the following protocol:

Phase	Voltage (V)	Duration (h)
Rehydration	50 µA/strip	12
Hold	500	2
Gradient	500-1000	2
Hold	1000	2
Gradient	1000-8000	2
Hold	8000	8
Total		**28**

The strips were taken off the strips holders and frozen overnight at -80°C prior to second dimension separation. The strips were taken off the freezer and thawed at RT before being equilibrated in order to re-solubilise the proteins and to reduce disulfide bonds (-SH-SH-). The strips were equilibrated gently in an orbital shaker, individually placed in cylindrical glass tubes containing 10 ml of SDS equilibration buffer with dithiothreitol (DTT) (2% w/v) for 15 min. Sulphide (-SH) groups were subsequently blocked with 10ml of solution containing SDS equilibration buffer and 2-iodoacetamide (2.5% w/v). Dithiothreitol (DTT) is a small-molecule redox reagent ($C_4H_{10}O_2S_2$) used to reduce the disulfide bonds of proteins and to prevent further intra and intermolecular disulfide bonds from forming between cysteine-residues of proteins. Since DTT cannot reduce buried (solvent-inaccessible) disulfide bonds, it has to be added to a urea containing solution (SDS equilibration buffer).

Iodoacetamide is an alkylating sulfhydryl reagent used for peptide mapping and binds covalently with cysteine, so the protein cannot form disulfide bonds. Iodoacetamide is highly toxic.

2.5.2. Second dimension

SDS-PAGE 2D acrylamide gels were made in-house using the Ettan Dalt II gel caster system (Amersham biotech, UK) and electrophoresis plates (1.5 mm-thick spacers). A 10% acrylamide solution was made immediately before use and poured into the caster. The gels were allowed to polymerise overnight.

The IPG gel strips were cut to size at both ends, taking care not to alter the hydrated gel and inserted between the two glass sheets of each plate and gently slid down until reaching the upper surface of the polymerise gel where they were sealed in place using a solution containing agarose (0.5% w/v) dissolved in SDS electrophoresis buffer by boiling it. Electrophoresis was performed using the Ettan Dalt™ II (Amersham biotech, UK) separation

unit. The gels were kept at a constant current of 5 watts per gel for 1 hour then subjected to a current of 80 watts per gel for up to 10-12 h or until the bromophenol dye used in the sealing solution had completed its run across the vertical axis of the gel. The electrophoresis separation unit was kept at 15°C.

2.5.3. Protein staining and detection

Available staining approaches include silver staining, Coomassie blue, Ponceau solution, amido black, India ink and radiolabelling (^{3}H, ^{14}C, ^{35}S, $^{32,33}P$ or ^{125}I). The gel cassettes were unloaded from the caster and the gels extracted and placed in separate plastic staining boxes. Proteins were fixed to the gel matrix using 40% ethanol / 10% acetic acid for 1 h at room temperature and then left in a solution containing 5% ethanol / 5% acetic overnight. Gels were stained with an optimised and adapted version of the protocol described by Hochstrasser *et al*. (Hochstrasser *et al.* 1988). This method has been proven to have high sensitivity and reduced intra and inter-experiment variability.

For preparative mass spectrometry silver staining was performed using a separate staining kit with slight modifications (Silver Plus one, Amerham biotech, UK) as follows:

	Step
Fix 40% ethanol/10% acetic acid	1 h
Soak in 5% ethanol/5% acetic acid	Overnight
Rinse in water	5 min
Soak in 0.5M Sodium acetate, 1% gluteraldehyde	30 min
Rinse in water	4x15 min
Soak in 0.05% Naphthalene sulphonic acid	2x30 min
Rinse in water	4x15 min
Silver stain For 12 gels (1.5 l): 12g silver, 750 ml ddH_2O, 20ml ammonium hydroxide and 3ml 10M sodium hydroxide)	25 min
Rinse in water	4x4 min
Develop (0.005% citric acid and 0.1% formaldehyde)	As required
Stop solution (5% tris and 2% acetic acid)	As spots start developing and background begins to appear
Storage solution (35% ethanol and 5% glycerol)	Months (if in cold room)

2.6. Gel scanning, image and statistical analysis for spot selection

Gels in all experiments were scanned immediately after being developed using a Images were scanned using a Bio-Rad GS-710 calibrated imaging densitometer and proprietary software (Bio-Rad, Seoul) and acquired as 12-bit .tiff files. Images of individual gels were cropped and saved in .tiff format for posterior image analysis which was performed using Progenesis SameSpots software (Nonlinear Dynamics, UK). Progenesis SameSpots software was selected on the basis that it was quicker and easier to use than MELANIE software, which had been successfully employed at the Lovestone lab previously. In addition, expression analysis studies using Progenesis SameSpots as part of the HUPO Reproducibility Study (http://www.fixingproteomics.org/advice/hupo_reproducibility_introduced.asp) have been shown to be reproducible cross-labs.

Such analysis involved the selection of a reference image as template for the overlaying and alignment of all the sample images in the experiment, followed by setting up detection parameters, filtering of irrelevant or wrongly detected spots and statistical analysis of established groups for comparison using fold change values and p-value (ANOVA), q-values (FDR), fold change and spot quality aspects such as definition and consistency.

Firstly, images were subjected to a process of quality control with feedback on intensity and saturation levels and corrective steps with in-built tools (flip, rotate, invert, and crop) before conducting the analysis (Figure 3).

One image per set was selected on the basis of overall quality as the reference image for the group against which the remaining images were to be aligned to. The alignment was performed step-wise using built-in visual tools (Figure 4) ('Whole Image' showing the two gels to be aligned overlapped with their spots represented in different colours (pink for the reference image and green for the image to be aligned to it); 'Vector editing' allowing for a smaller area of the gel to be examined in detail and to add manual and automatic vectors linking the same spots in both gels; 'Transition' for obtaining a dynamic representation of both gels fading in and out and facilitating the visualization of changes in positioning that needed to be corrected by the use of vectors. This is performed until the only changes shown are those of signal intensity in a fixed spatial location with no lateral movement; 'Checkerboard', or a composite of both gels used as a proxy measure of alignment accuracy).

An average of 50 vectors per image was added manually to known protein patterns. Automatic vectors were subsequently added by the software using an image recognition algorithm to correct the alterations the gel matrix contained and the different spatial locations

of proteins relative to their theoretical points of anchorage. Images were then 'aligned' (Figure 5). All the identified spots underwent an analysis process that included spot detection, background subtraction, normalisation and matching. The resulting spots were outlined and could have been filtered and deleted according to average normalised volume, area or by manual selection (Figure 6). All the spots considered as such by the program were brought forward for the next stage of the image analysis. Images were grouped according to the experimental design set up in order to conduct the comparative analysis of expression. The individual images were grouped according to treatment arms (control, haloperidol, citalopram). Different comparisons were performed (3 groups ANOVA and 2 groups non-parametric testing (haloperidol vs. control; citalopram vs. control and haloperidol vs. citalopram).

A list of spots was presented for review (Figure 6). Spots were statistically ordered according to p-value from the one way ANOVA and fold change. Each spot was assigned a unique identifying number that was maintained throughout the experiment and on every analysis performed regardless of the grouping the images are subjected to. The visual aids displayed in this interface (i.e. expression profile, 3D and 2D montage and full image outline) allowed for an effective selection of potentially relevant spots (Figure 7). Spots could also be tagged to create sub-sets within the main analysis, using complementary data such as phenotypic information, for instance. Notes were also added to each spot to be included in the final report.

The statistical analysis was initially performed using the built-in Progenesis Stats software (a statistical resource containing Principal Component Analysis (PCA), Correlation Analysis, Power Analysis and False Discovery Rate and Q-values) (Figure 8) and verification of the results obtained was subsequently conducted using SPSS v15 and v16. No missing values were yielded by Progenesis SameSpots and Progenesis Stats used univariate and multivariate statistical techniques to further interrogate this complex data in a continuous work-flow.

Principal Component Analysis (PCA) is a statistical technique not too dissimilar to factor analysis used to find common variation patterns in multiple data sets. This is achieved by simplifying the observed variables into a smaller number of artificial variables called principal components. These components account for a high proportion of the variance in the observed variables. The underlying assumption being that some of the information yielded is redundant as they are purported to measure the same construct. PCA was thus performed to provide a simplified representation of the multidimensional data by grouping individual images and to identify potential outliers.

Correlation Analysis is another multivariate unsupervised approach that provides a summary of protein activity and assist in identifying features pertaining to the same biological processes and pathways. Power analysis is calculated based on the sample size and is useful in pilot studies to verify the number of replicates needed to obtain 80% statistical power. False Discovery Rate and Q-values give an indication of the number of the expected proportion of false positives that can be expected if that feature's p-value is chosen as the significant threshold.

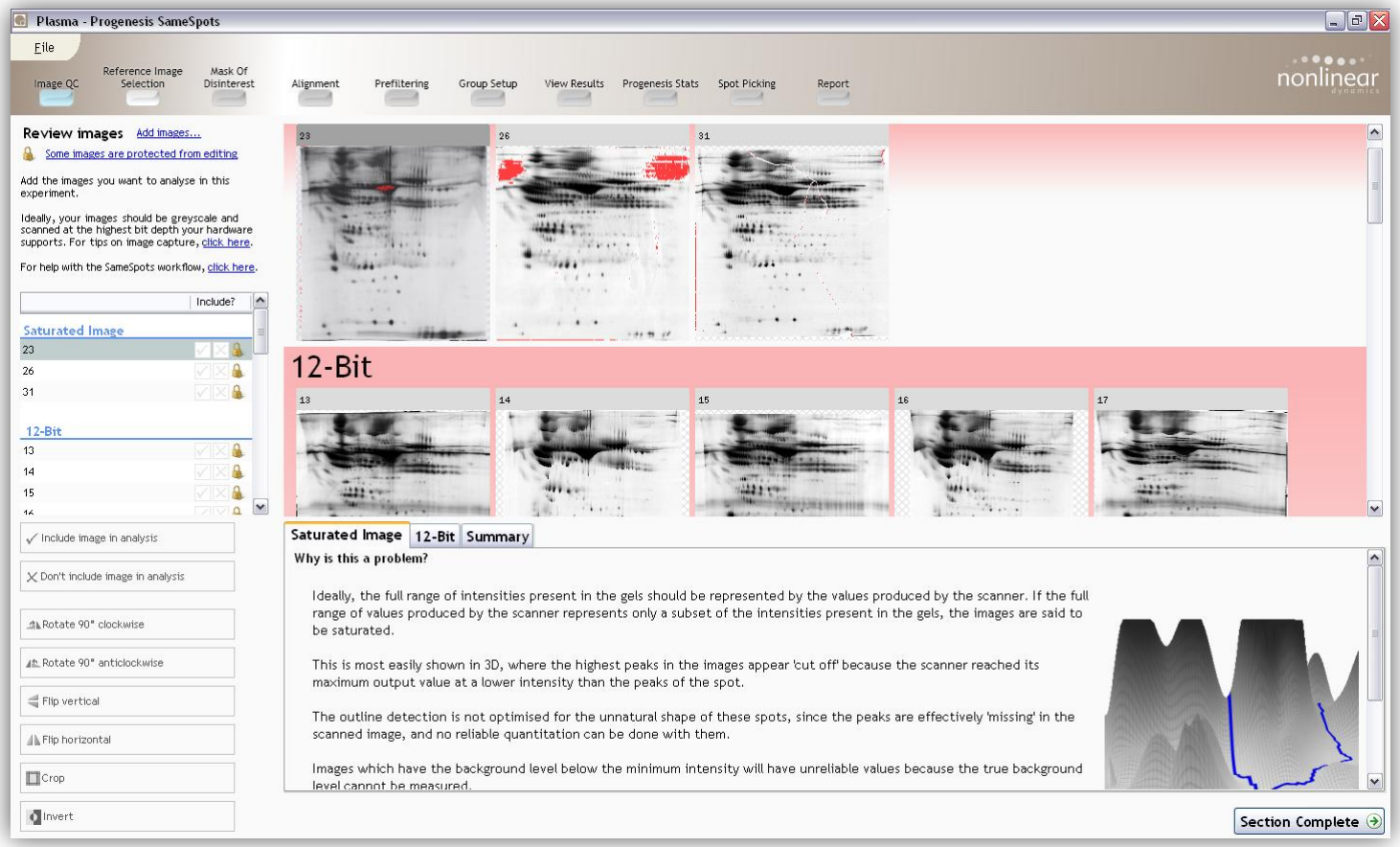

Figure 3. Screen shot of Image Quality Control stage in the plasma study.

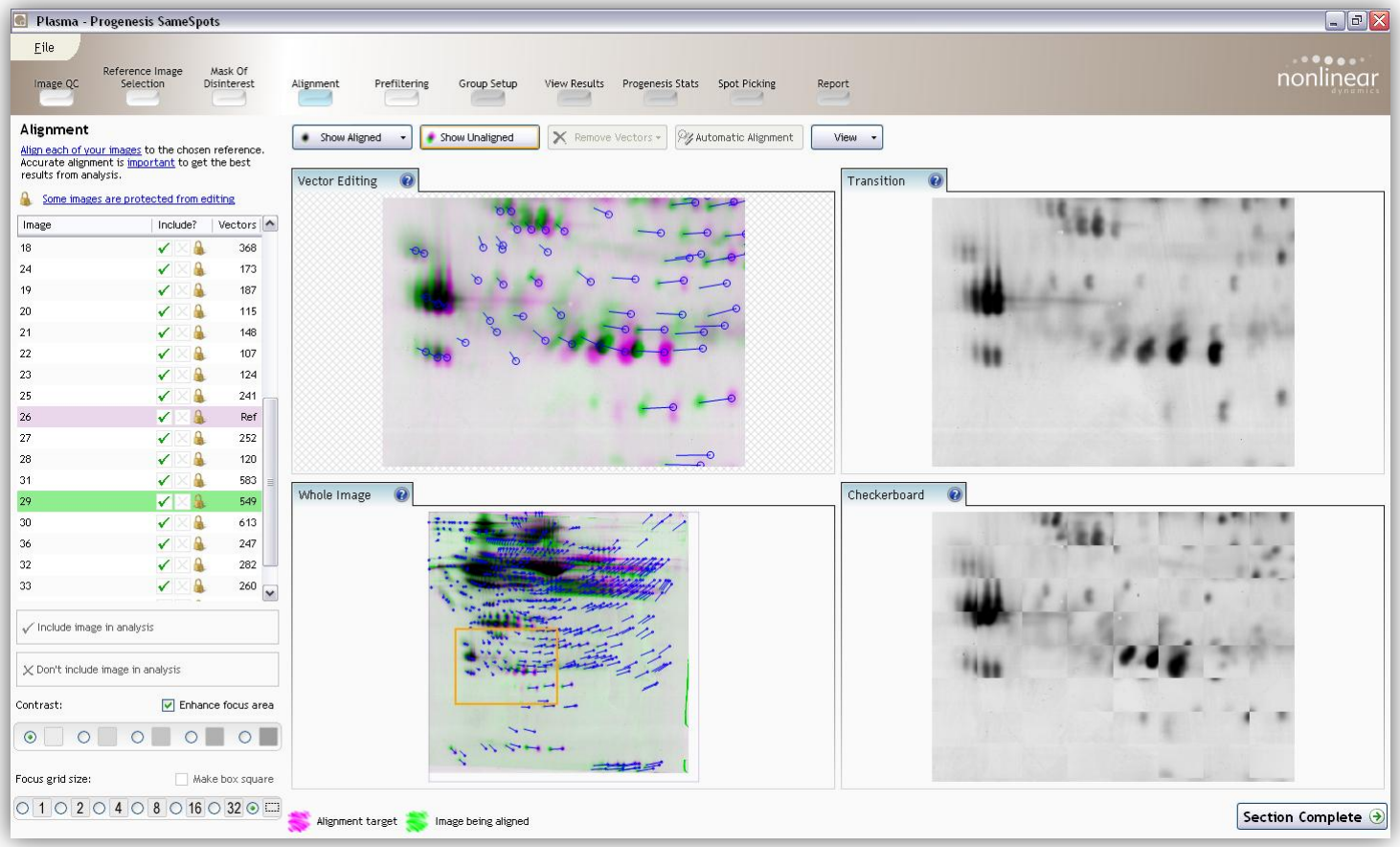

Figure 4. Screen shot of Progenesis SameSpots visual tools used for alignment.

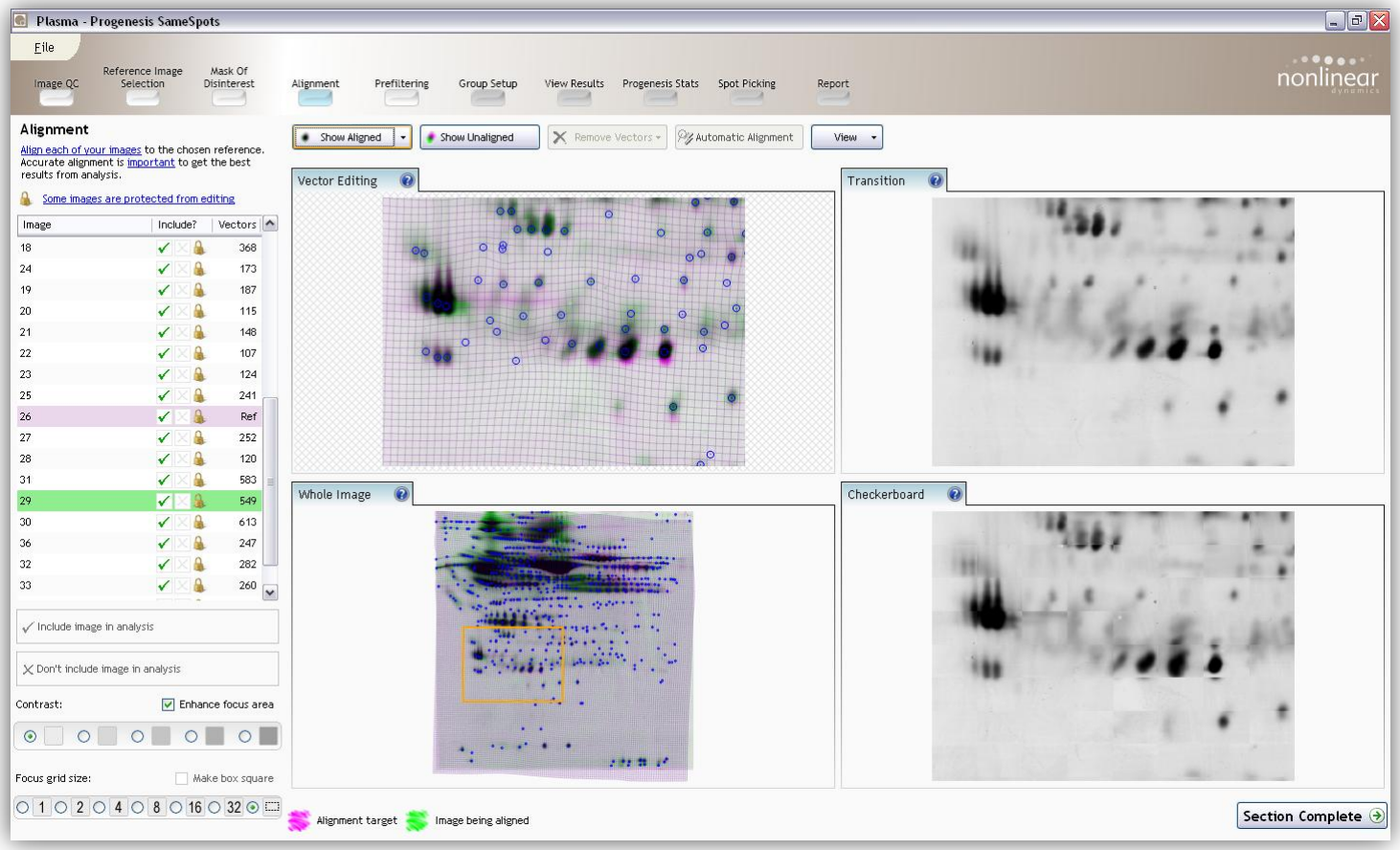

Figure 5. Screen shot of Alignment stage (images aligned). Note how the Checkboard view resembles the Transition view.

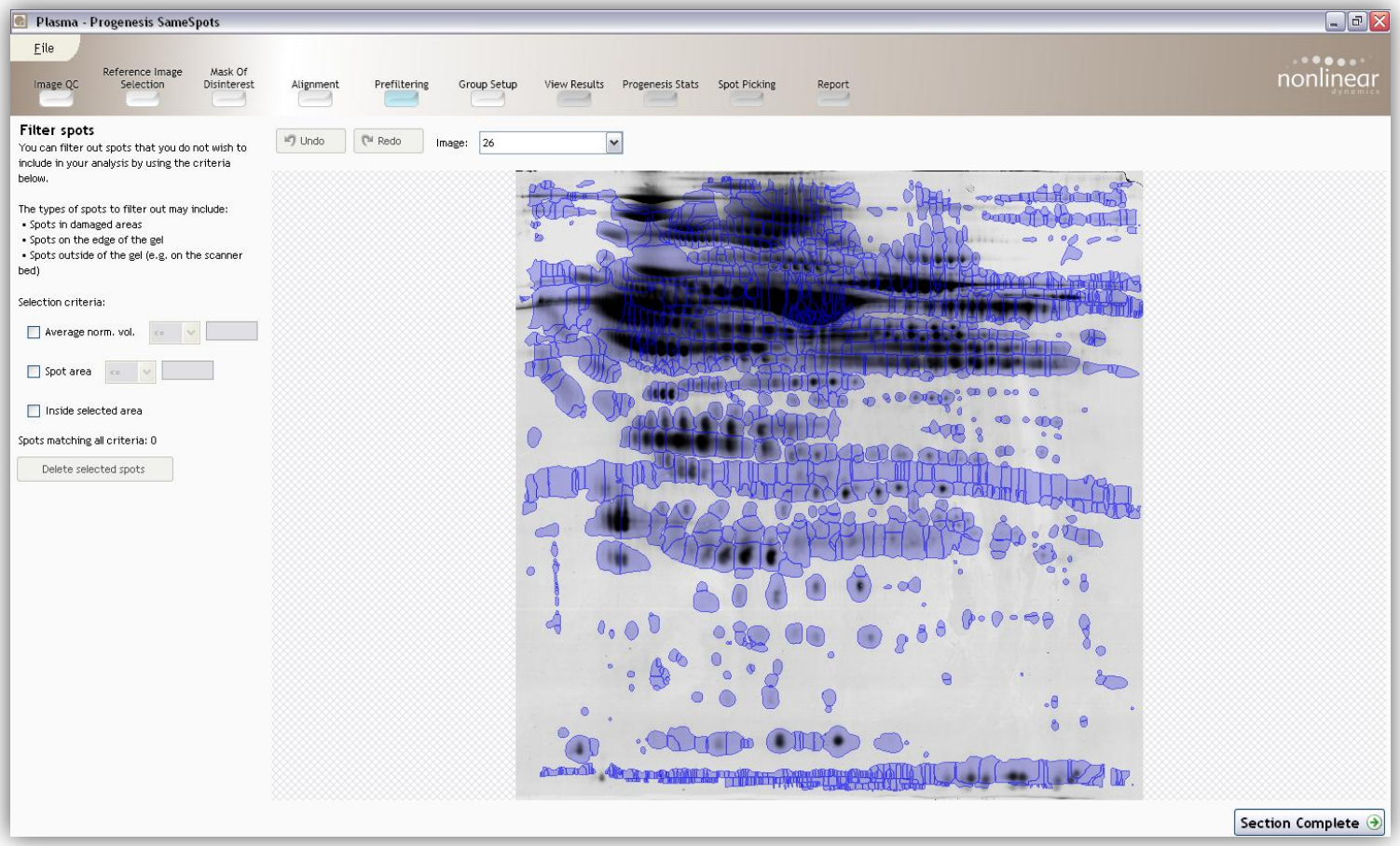

Figure 6. Screen shot of the Prefiltering stage with all the spots identified outlined and ready for manual curation and analysis.

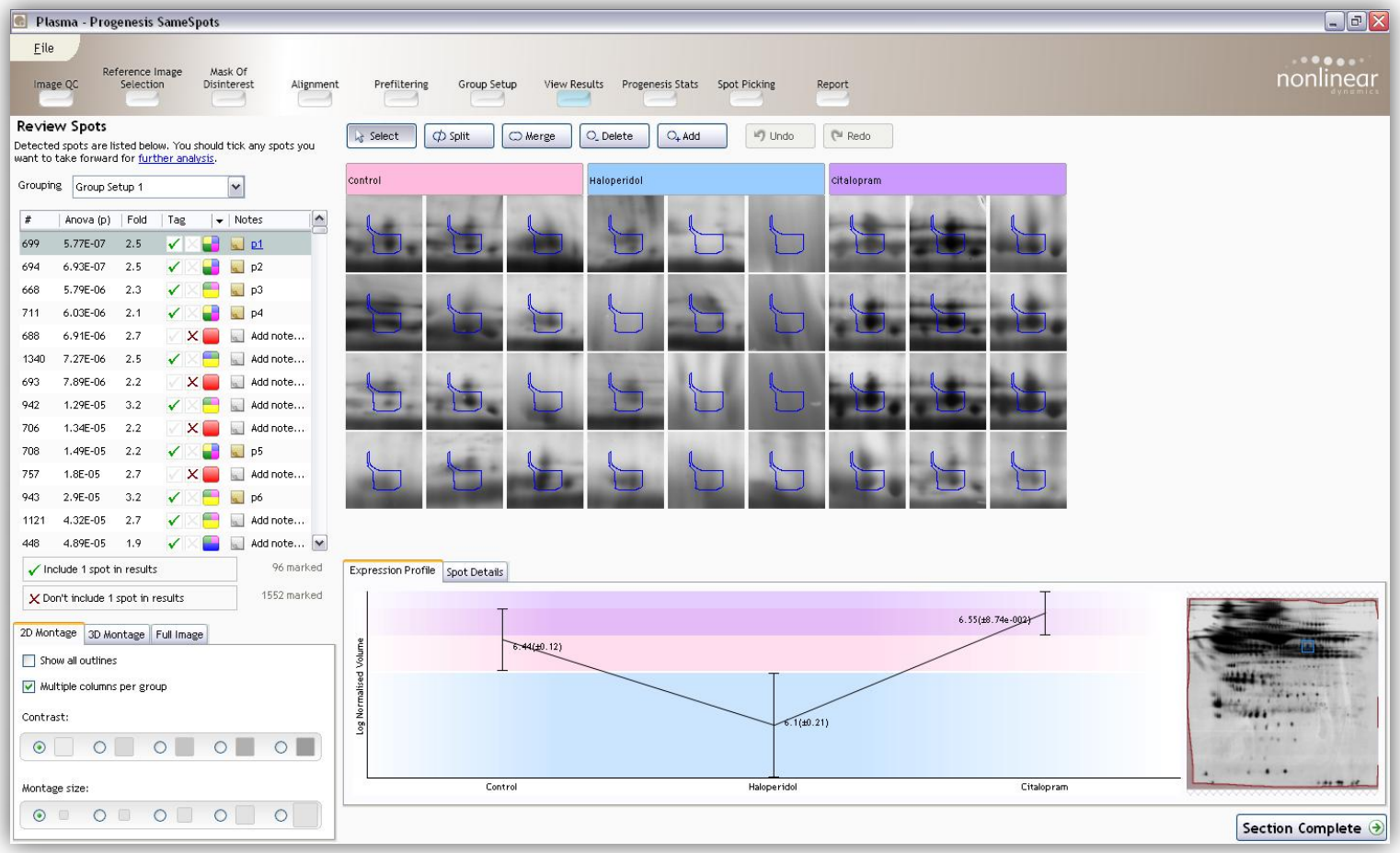

Figure 7. Screen shot of the View results stage with all the spots identified outlined and ready for manual curation and analysis.

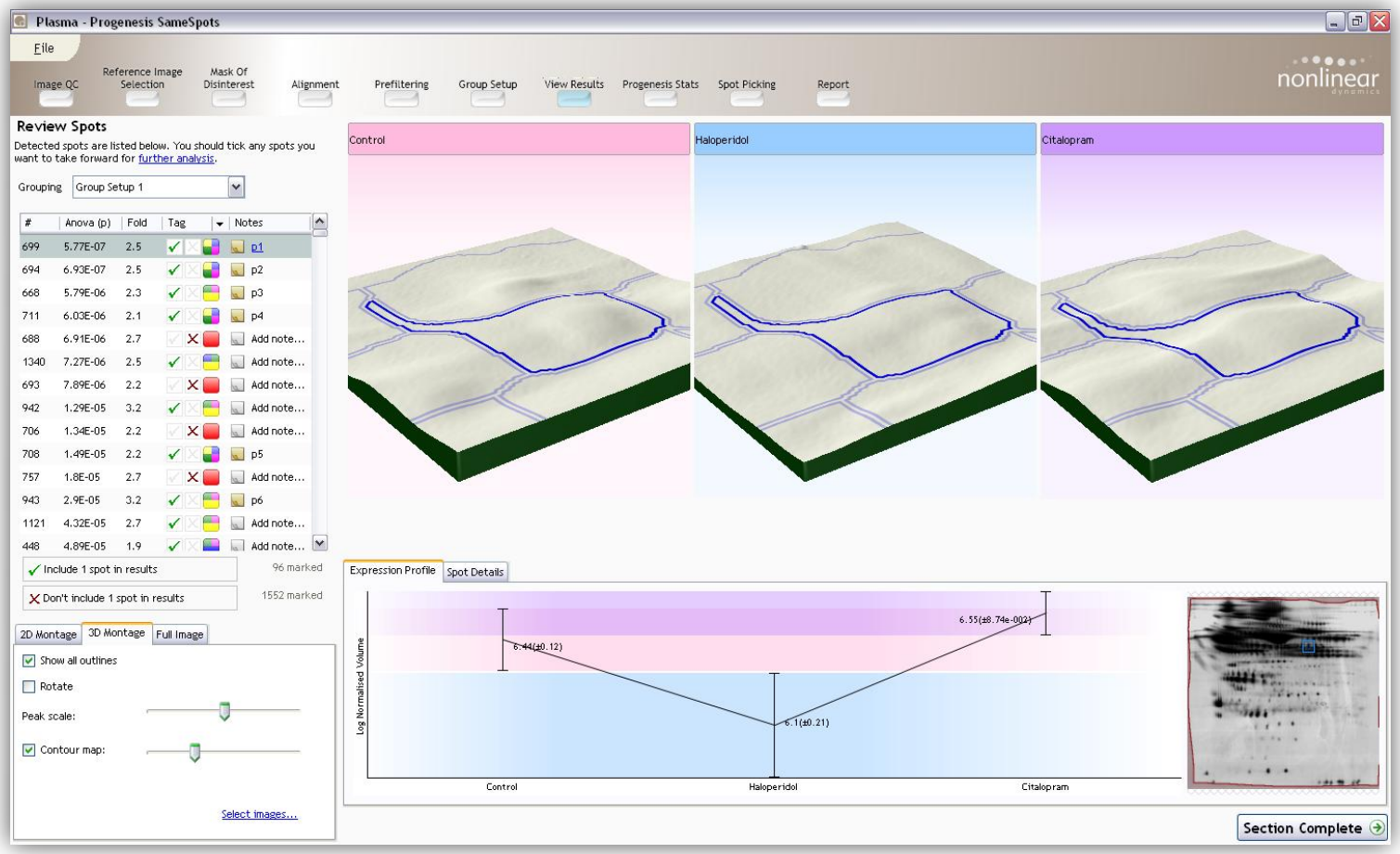

Figure 8. Screen shot of the View results stage showing the 3D montage view.

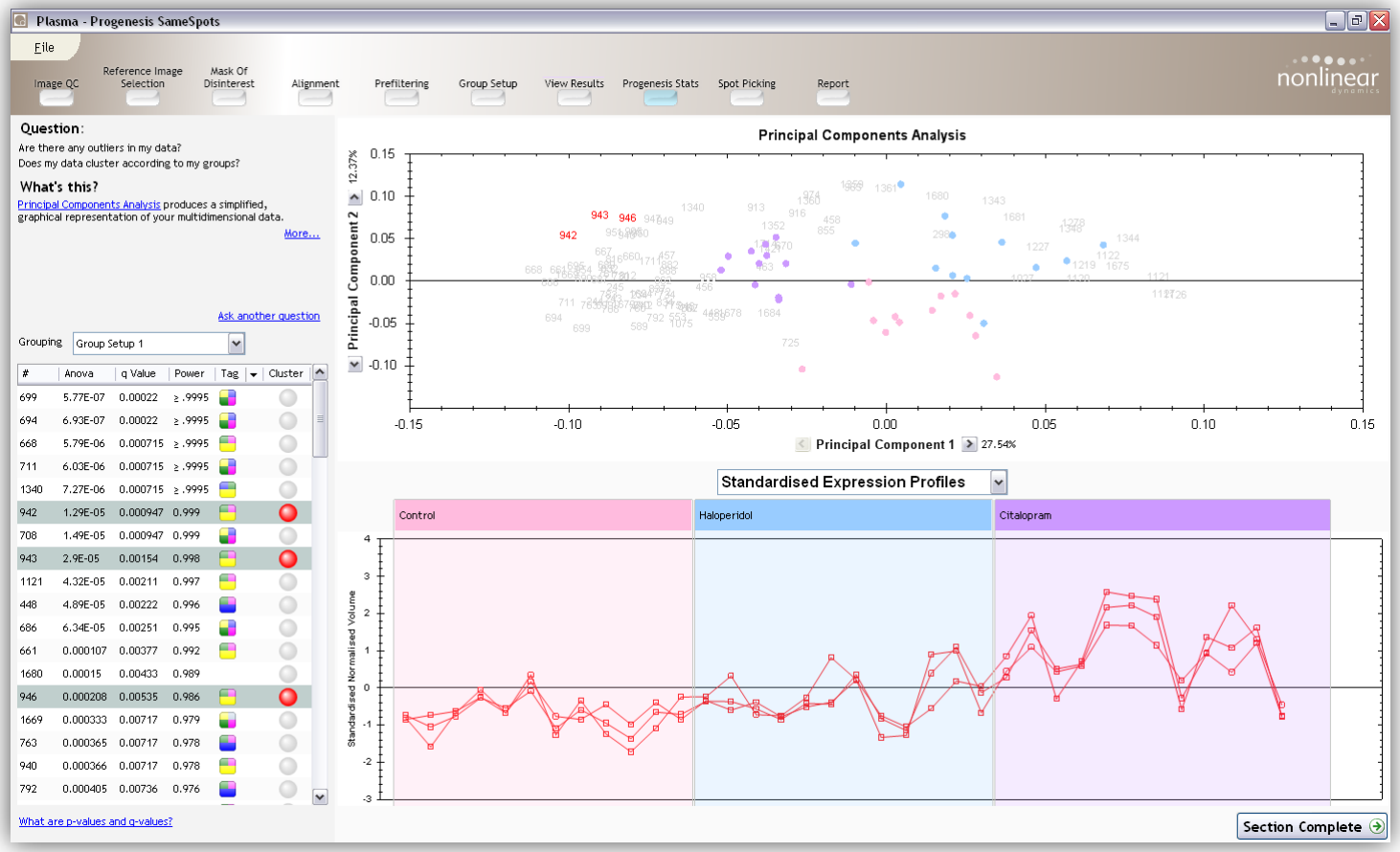

Figure 9. Screen shot of the Progenesis Stats screen showing the principal component analysis and Standard expression profile of selected spots (highlighted).

2.7. Protein identification (LC/MS/MS)

Gels were stained with modified Plus One silver stain kit (Amersham, UK) to make silver staining compatible with MS procedures and selected spots were manually excised from 2-DGE preparatory gels containing 300 μg of protein.

The spots were identified independently by the author and two experienced postdoctoral researchers and excised carefully using a retroillumination and a 200 or 1000 μl pipette tip with its end cut off. The gel fragments were suspended in ddH_2O and subsequently taken to King's Proteomics Facility and processed by Steve Lynham.

The gel matrix was subjected to in-gel reduction, alkylation and digestion with trypsin. Cysteine residues were reduced with DTT and derivatised by treatment with iodoacetamide to form stable carbamidomethyl derivatives. Trypsin digestion was carried out overnight at room temperature after initial 1 h incubation at 37°C. Peptides were extracted from the gel pieces by a series of acetonitrile and aqueous washes. The extract was pooled with the initial supernatant and lyophilised. Each sample was then re-suspended in 23 μl of 50 mM ammonium bicarbonate prior to performing peptide mass fingerprinting, in where a series of peptides obtained from native proteins using residue-specific enzymes have their individual masses measured by spectrometric techniques and then examined against theoretical peptide libraries, yielding a list of likely protein identifications, and analysed by liquid chromatography tandem mass spectrometry (LC/MS/MS).

Chromatographic separations were performed using an Ultimate LC system (Dionex, UK). Peptides were resolved by reversed phase chromatography on a 75 μm C18 PepMap column using a three-step linear gradient of 0-48% acetonitrile/0.1% formic acid over 30 minutes at a flow rate of 200 nl/min. Peptides were ionised by electrospray ionisation using a Z-spray source fitted to a QTof-micro (Waters Corp.) operating under MassLynx v4.0. The instrument was set to run in automated switching mode, selecting precursor ions based on their intensity, for sequencing by collision-induced fragmentation. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the m/z and the charge state of the peptide.

The mass spectral data was processed into peak lists using ProteinLynx Global Server V2.2.5 with the following processing parameters: smoothing by Savitzky-Golay method, 2 iterations, 4 channels; peak centroiding top 80%, no deisotoping or background subtraction. Proteins were identified by searching the peak lists against the Uniprot database (version Aug_2011) using Mascot software V2.2 (Matrix Science, UK). Parameter specifications for database searching were as follows: Precursor ion mass tolerance 1.2 Da, fragment ion mass

tolerance 0.6 Da, up to three missed cleavages (trypsin), variable modifications: cysteine carbamidomethylation, methionine oxidation and pyroglutamisation of N-terminal glutamine residues. Sequence information was obtained for all the peptides included in the results.

2.7.1. Bioinformatics

The mass spectral data was processed into peptide peak lists and searched against the Swiss Prot Database (current as of June 2005) using Mascot software (Matrix Science, UK). Assumptions included peptide mass tolerance of 1 Da and fragmentation tolerance of 0.5 Da, a maximum of two mis-cleavages and MOWSE algorithm score of 95% confidence. Where reported peptide matches are below 95% confidence limits, each peptide was visually verified before inclusion. Proteins with 3 or more peptides above the confidence limit are considered to be real and not a false positive. Proteins with less than 3 significant peptides were subjected to a search in a reverse sequence database (Swiss Prot in reverse). To add further confidence the peptide sequence for each protein were submitted to an NCBI protein-protein BLAST search to ascertain that the peptide sequence is unique to that protein. Carbamidomethylation and oxidation were set as variable modifications within the searching parameters.

For the statistical analysis raw integrated optical density (IOD) spot data from each of the images into SPSS (v 11) and Progenesis Stat package. Normalized spot data is obtained by Progenesis. The OD of individual spots was normalized by the software using the background density as a measure of exposure and developing time.

2.7.2. Identification of proteins that differentiate between cases and controls

Statistical analysis was performed using SPSS (v11), and Progenesis SameSpots packages. It is known that biological protein expression data obtained from a 2-DGE is generally not distributed normally. Therefore integrated optical densities of spots on 2D gels were tested for normality using the Shapiro-Wilko test. Spots examined were not normally distributed therefore non-parametric tests (Mann-Whitney) combined with false discovery rate analysis as correction for multiple comparison testing was applied. The assessed mean difference between patients and controls was applied to each spot using Mann-Whitney. The p-values for the null hypothesis of no mean differences were saved sorted by increasing p value and ranked. By ranking the significant protein abundances it allowed the application of a further test False Discovery Rate (FDR). False discovery rate or FDR is a calculation of the proportion of false positives among all positives. This test was first introduced by Benjamini and Hochberg in

1995. For example if the choice of threshold is set at FDR of 0.05 (5%), one would expect, 5% falsely induced protein spot expression. In addition it was expected that 95% of the rejected protein change to be truly induced (Benjamini et al., 2001). Following this, the proteins in the selected spots were then identified using tandem liquid mass spectrometry (LC/MS/MS) as described earlier.

2.8. Validation

2.8.1. Serotonin ELISA in rat plasma and brain

In order to establish that the surgical procedure had successfully delivered the antipsychotic and antidepressant agents, it was decided to measure serotonin levels in brain and plasma as both agents are known to affect serotonergic transmission. We used the Serotonin ELISA Fast Track (Labor Diagnostika Nord) to measure serotonin levels in plasma and the Serotonin Research ELISA (Labor Diagnostika Nord) to measure the levels of serotonin in brain. Plasma samples were stored at -80°C were thawed on ice until they reached room temperature (RT) prior to being used. A volume of 25 µl per sample was used in the assay. Brain samples, which were preserved at -80°C were thawed on ice, eluted in lysis buffer and diluted 1:10 prior to use. A volume of 100 µl per sample was used.

2.8.2. Western blot in human plasma

Western blot (protein immunoblot) was used to detect the specific proteins of interest in an independent sample of subjects. Briefly, the Western blot method is used to separate proteins according to molecular weight by means of gel electrophoresis. The separated proteins are then transferred to a membrane where they are incubated with specific antibodies. Plasma proteins were resolved in 10% (w/v) polyacrylamide SDS-PAGE using the Gel unit vertical complete for precast or handcast gels up to 200mm x 100mm (Fisherbrand, FB69602). Plasma was diluted at 1:50 in PBS (loading volume was 10 µl per well). Gels were run at constant voltage (200V, 3.00 A and 300 W) with a Bio-Rad PowerPac 300 until the dye reached the bottom end of the gel (1-2 hours).Once protein separation occurred in the acrylamide gels, the proteins were transferred to 17x6 cm pieces of 0.45 µm nitrocellulose membrane (Schleicher & Scheull) in order to be probed with specific antibodies. A Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) (Fig3) was used and the following sequence was assembled in the vertical panels: cathode (black cover) (the terminal from where the current flows out, usually the negative pole)/6 Whatman 3MM filter papers soaked in transfer buffer/Gel-Nitrocellulose membrane-6 Whatman 3MM filter papers soaked in transfer buffer/anode (transparent cover)

(the terminal where the current flows in from the outside, usually the positive pole). Proteins migrate from the gel into the nitrocellulose membrane as they are negatively charged as a result of the SDS.

A current of constant voltage of 100 V was then applied for 1 hour. Following the transfer, the membrane was incubated with Ponceau solution for 1 min to determine the efficiency of the protein transfer and subsequently blocked to reduce non-specific binding by using a 5% non-fat milk 0.01 M PBS solution.

Membranes were then incubated overnight at 4°C in blocking buffer with the primary antibody at an optimized dilution for detection based on manufacturer's protocol.

The following morning, the membranes were washed in 0.1% PBS-Tween (3x5 min) and placed in a white, non-transparent box containing 10 ml of 0.1% PBS Tween + 5% milk for 30 min at room temperature. Secondary antibodies were diluted in 5% Milk PBSTween solution (1:6000) in a Falcon tube protected from light with foil paper. The 0.1% PBSTween+5% milk of the boxes containing the membranes was replaced with the secondary AB solution and the boxes were placed in an orbital rocker for 1 hour at RT. The membranes were washed on PBSTween 3x 5 minutes and scanned using a LI-Cor Odyssey scanner and proprietary software was used for fluorescence detection.

The blots were run in triplicate. Fluorencence signal intensities were individually measured (K counts). The average was calculated and normalised as follows: individual samples were performed across three gels. Mean optical density was calculated per each subject and a total average was calculated for the entire group (i.e. cases and controls). Subsequently, the group average value was used to correct the measure of the individual average mean. An independent samples t-test for equality of means was applied using SPSS v16.0.

2.8.3. S100B ELISA

S100B was measured using Merck/EMD Millipore's Human S100B ELISA kit (Cat. No. EZHS100B-33K) designed to allow to measure levels of S100B in cerebrospinal fluid (CSF), serum or plasma (heparin or EDTA). This kit was validated in human samples, but the assay is cross-reactive in multiple species, including bovine, hamster, horse, monkey, mouse, pig, rabbit and rat.

The ELISA assay was performed in a 96-well plate according to the manufacturers instructions supplied with Human S100B ELISA 96-well plate (Cat. #EZHS100B-33K). All plasma samples were used as neat as no prior dilution was required. Briefly, the plate was rinsed with 300 µl diluted wash buffer. Subsequently, 50 µl of controls and standards were added to the

appropriate wells. 50 µl plasma samples were added to the appropriate wells, with 50 µl of assay buffer alone to the blank wells. Plate was sealed and incubated 2hr on an orbital shaker at room temperature (RT).

The plate was washed 5 times with wash 300 µl buffer; 100 µl detection antibodies was added to all wells and incubated for 1.5 hr on an orbital shaker at RT. Again, the plate was washed 5 times with wash 300µl buffer; 100µl enzyme solution added and incubated for 30min at RT on a shaker. Once more the plate was washed 5 times with wash 300µl buffer; 100µl substrate solution was added to all wells incubated for 5-25min depending on colour change. The reaction was stopped with 100µl stop solution and absorbance read in a plate reader set at 450nM. All plasma samples were performed in duplicate.

CHAPTER 3: Examining the effects of DISC1 knockdown on the proteome of human neural progenitor cells

3.1. Rationale and aims

It is widely accepted that SCZ is a polygenic disorder for which the underlying biological pathways remain at best speculative. A (1;11)(q42;q14.3) chromosomal translocation affecting the disrupted-in-schizophrenia-1 (DISC1) gene has been found to co-segregate with SCZ and other major psychiatric disorders in a large Scottish pedigree (Blackwood *et al.* 2001; Hennah *et al.* 2003). Linkage and association studies suggest that variation in DISC1 may also confer risk for psychiatric disorders in general populations (Ekelund *et al.* 2001; Hamshere *et al.* 2005; Hennah *et al.* 2003; Hodgkinson *et al.* 2004; Macgregor *et al.* 2004; Schosser *et al.* 2010; Schumacher *et al.* 2009). DISC1 is highly expressed in the cerebral cortex, hippocampus and amygdala (Millar *et al.* 2000) and has been shown to be involved in neural progenitor cell proliferation and differentiation, via interaction with glycogen synthase kinase 3 (GSK3) (Mao *et al.* 2009), as well as neural migration and neurite outgrowth (Duan *et al.* 2007; Ishizuka *et al.* 2011; Kamiya *et al.* 2005).

In this study, 2DPAGE and MS was used to determine protein expression changes resulting from DISC1 knockdown in human cortical neural progenitor cells (CTXOE03) using RNA interference (RNAi). This was intended to model the haploinsufficiency of DISC1 that is thought to mediate the pathogenic effects of the t(1;11)(q42;q14.3) translocation (Millar *et al.* 2005).

The aim of this study was to explore, using a classical proteomic approach, the molecular and cell signalling pathways that may contribute to the signalling alterations that are thought to be responsible for the varied array of symptoms of SCZ.

The culture, transfection and harvesting of CTXOE03 cells was done by Sinead Kinsella, an MSc in Neuroscience student in Dr N. Bray's lab in the Department of Neuroscience, Institute of Psychiatry, who used some of these data for her dissertation.

3.1.1. DISC1

Long- term psychiatric follow up studies of this Scottish family showed that 18 of 29 carriers of the (1;11)(q42;q14.3) chromosomal translocation were diagnosed with a major mental illness (Blackwood and Muir, 2004; Chubb *et al.* 2008). Association studies have linked DISC1 to SCZ (Hennah *et al.* 2003) and a genomewide screen performed in an isolated Finnish population revealed several putative loci in four chromosomal regions (one of them was 1q32.2-q41) predisposing to SCZ (Hovatta *et al.* 1999), although it is unlikely that common DISC1 SNP alleles account for a substantial proportion of the genetic risk of SCZ, according to recent study in a large (837 cases and 1473 controls) Scandinavian case-control sample (Saetre *et al.* 2008).

A second gene, named disrupted-in-SCZ-2 (DISC2), encoding an RNA gene antisense to DISC 1 was also found to be affected by this translocation (Chubb *et al.* 2008).

DISC1 encodes a scaffold protein with multiple coiled coil motifs which is located in the nucleus, cytoplasm and mitochondria and is highly expressed during brain development. This protein is likely to have multiple function (Brandon *et al.* 2009; Chubb *et al.* 2008) and three isoforms have been identified so far within molecular weight range of 70-85 kDa (James *et al.* 2004). The DISC1 protein may play a role in nucleus-centrosome association, neuronal proliferation, differentiation and migration, hippocampal formation and cortical layering, all of which are affected in SCZ (Brandon *et al.* 2004; Millar *et al.* 2005). DISC1 has been shown to interact with a range of cytoskeletal proteins some of which, like NudeE-like (NUDEL) are associated with cortical development and linked to the lissencephaly gene (LIS-1), responsible for a rare cortical developmental disorder in which abhorrent neuronal migration in uterus results in lack of development of brain gyri and sulci conferring it a smooth aspect macroscopically (Ozeki *et al.* 2003).

In addition, DISC1 appears to be a component of the microtubule-associated dynein motor complex and is essential for maintaining the complex at the centrosome (Kamiya *et al.* 2005). As a result, depletion of DISC1 protein impairs neurite outgrowth. This cellular alteration leads to abhorrent cortical circuitries. It has been shown that mice displayed behavioural abnormalities post-puberty when DISC1 was knockdown in pyramidal neurons in the prefrontal cortex in utero (Niwa *et al.* 2010).

DISC1 deficiency could be mediated by two different mechanisms: 1) haplo-insufficiency, by which two functional copies of the gene are required for the normal expression levels of the protein; and 2) dominant-negative, by which the abnormal product derived from the mutant allele inhibits the wild-type product. Based on this, genetically engineered transgenic mice have been used to study the downstream effects of DISC1 depletion in mammals.

A strand of transgenic mice generated using a bacterial artificial chromosome (BAC) and expressing 2 copies of truncated DISC1 exhibited increased immobility and reduced vocalization in depression-related tests, and impairment in conditioning of latent inhibition. The animals had enlarged lateral ventricles, reduced cerebral cortex, partial agenesis of the corpus callosum, and thinning of layers II/III with reduced neural proliferation at midneurogenesis. In addition, parvalbumin GABAergic neurons were reduced in the hippocampus and medial prefrontal cortex. It was also shown that cultured transgenic neurons grew fewer and had shorter neurites (Shen *et al.* 2008).

Since expression of dominant-negative proteins has been frequently used in animal models to achieve partial loss of function for other proteins (Oike *et al.* 1999), in another animal model, a dominant-negative form of DISC1 (DN-DISC1) was expressed under the control of the promoter for the α-isoform of calcium/calmodulin-dependent kinase II (αCaMKII). The resulting transgenic mice were shown to display abnormalities consistent with findings in severe SCZ like enlarged lateral ventricles, particularly on the left side, selective reduction of parvalbumin (a marker for interneuron deficit) in the cortex, and behavioural abnormalities including hyperactivity, disturbance in sensorymotor gating and olfactory-associated behaviour and an anhedonia/depression-like deficit (Hikida *et al.* 2007).

DISC1 has shown to have a role in the regulation of synapse maturation (Brandon *et al.* 2009; Camargo *et al.* 2007) and of spine density, which had been previously reported to be decreased in the dorsolateral prefrontal cortex layer 3 pyramidal neurons of patients with SCZ (Glantz and Lewis, 2000).

Despite all this evidence, the mechanism by which DISC1 confers its susceptibility to psychosis remains largely unknown. It has recently been suggested that DISC1 interacts with Phosphodiesterase 4B (PDE4B) and that elevation of cAMP leads to dissociation of TPDE4B from DISC1 and an increase in its activity, which in turn inactivates cAMP (Millar *et al.* 2005). DISC1 is involved in the Wnt receptor signaling pathway (Figure 10), which also contains DRD2 and GSK3-β. This signalling pathway is a target for lithium and it is involved in carcinogenesis, neurodevelopment, and axon guidance, regulation of cytoskeleton and of intracellular calcium levels. Downregulation of DISC1 has been shown to lead to a decrease in the cytosolic level of β–catenin (Brandon *et al.* 2009; Mao *et al.* 2009) which is involved in the maintenance of adherens junctions in epithelial cells and in anchoring the actin cytoskeleton, transmitting the contact inhibition signal that causes cells to stop dividing. β–catenin is a transcription factor and a substrate of GSK3-β, which is a crucial node for mediating neurogenesis, neuronal polarization and axon growth during brain development (Hur and Zhou, 2010).

There is evidence to suggest that through physical interaction with GSK3- β, DISC1 prevents GSK3-β from phosphorylating β–catenin (Mao *et al.* 2009). The inhibition of GSK3- β-mediated-phosphorylation of β–catenin allows β–catenin to enter the cell nucleus, interact with transcription factors and regulate gene transcription.

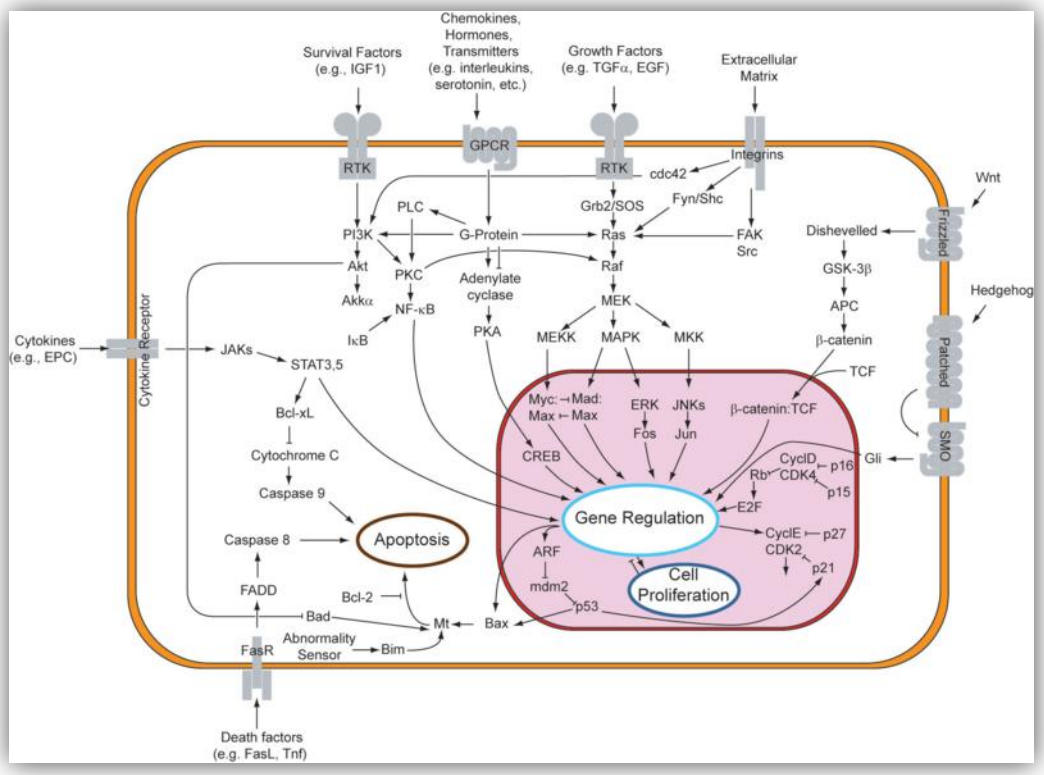

Figure 10. Wnt signalling pathway.

In a study on patients diagnosed with Major Depressive Disorder (MDD), DISC1 knockdown resulted in suppression of phosphorylation of ERK and Akt and reduction brain gray matter volume in cingulate cortex in individuals carrying it compared to Ser/Ser704 subjects (Hashimoto *et al.* 2006).

Thus, DISC1 was selected for this study with the purpose of examining the underlying molecular pathways mediating symptomatology in SCZ as it has provided the most pragmatic findings for determining etiologic mechanisms and understanding of SCZ. Discovery-phase experiments using two dimensional gel electrophoresis (2DPAGE) and mass spectrometry-based proteomic analysis of plasma were performed as they had been successfully conducted by our group previously to study Alzheimer's disease (Hye *et al.* 2006), and also more recently in the context of non-demented subjects (Guntert *et al.* 2010).

3.2. Materials and methods

3.2.1. Culturing of CTXOE03 cells

All cell culture work was carried out by MS Sinéad Kinsella in Dr Nicholas Bray's lab under the tissue culture hood in sterile conditions. A detailed description can be found in the Methods chapter.

The CTXOE03 neural progenitor cell line were grown on laminin at 37°C with DMEM F12 media supplemented with human albumin serum, transferrin, putrescine DiHCl, insulin, progesterone, L-glutamine, sodium selenite, bFGF, EGF, and 4-OHT. RNAi transfection was carried out using the NTER system (Sigma). There were 4 RNAi conditions - 'Control RNAi', 'RNAi1', 'RNAi2' and 'No RNAi'. Cells were harvested six days after initial siRNA transfection. RNA extraction was performed using Tri Reagent (Ambion). cDNA synthesis was carried out using Superscript III (Invitrogen), following DNase treatment of extracted total RNA. Analysis was carried out using MJ Opticon software from Bio-Rad. Average values of duplicates for DISC1 were divided by average of duplicate values of the POLR3C housekeeping gene for each cDNA sample. Expression values were compared between RNAi1 vs. Control RNAi, and RNAi2 vs. Control RNAi using 2-tailed t-tests.

3.2.2. Protein analysis

Nanodrop (ND-1000) was used to measure protein concentration in samples. The desired volume, containing 50 µg of protein, was calculated for each sample from protein concentration measurements. Individual gels corresponded to each one of the six samples

(designated A-F) in each of the four RNAi conditions (RNAi1, RNAi2, Control RNAi and No RNAi). Twenty-four gels in total were run in 2 batches of 12 gels each to minimise experimental error due to the batch effect.

The first set of gels run contained RNAi1, RNAi2, Control RNAi and No RNAi samples A-C; and the second batch contained RNAi1, RNAi2, Control RNAi and No RNAi samples D-F. 350 µl Rehydration buffer was added to the appropriate sample volume. Gels were run in batches of 12. Individual samples were dissolved in IPG rehydration buffer and 350 µl total volumes was loaded in 18cm Immobiline DryStrips (pH 3-11). Proteins were separated according to individual isoelectric point (PI) using the IPGphor™ (Amersham Bioscience) platform and subsequently equilibrated with dithiothreitol (DTT) and 2-Iodoacetamide

The second dimension (separation of proteins by size) was performed using SDS-PAGE 2D acrylamide gels on an electrophoresis Ettan Dalt™ II (Amersham Biosciences) separation unit. Proteins were fixed to the gel matrix and stained with an optimised and adapted version of the protocol described by Hochstrasser *et al*. (Hochstrasser *et al.* 1988). Gels were scanned immediately after being developed and the subsequent image analysis was performed using Progenesis SameSpots software (Nonlinear Dynamics, UK) version 3.3.3420.25059.

Following the analysis of the images, preparatory gels were made with 300 µg protein and selected spots were excised from the gel matrix, suspended in ddH_2O and taken to King's College London Proteomics facility to be processed by Steve Lynham. The gel fragments were subjected to in-gel reduction, alkylation and digestion with trypsin prior to performing peptide mass fingerprinting and analysed by liquid chromatography tandem mass spectrometry (LC/MS/MS).

3.3. Results

3.3.1. Transfection of CTXOE03 Cells and DISC1 knockdown

From the images taken (Figure 11) it can be seen that successful uptake of the red fluorescent oligonucleotide into CTXOE03 cells took place and can be assumed that the transfection of the samples was successful. Statistical analysis of data from the qPCR reported DISC1 to be successfully knocked down in the CTXOE03 cells following treatment with two different siRNAs specific for DISC1. Analysis of this qPCR data showed a 35% reduction in DISC1 expression for the samples treated with RNAi1 ($p = .046$, SEM = 0.09) and a 38% reduction in DISC1 expression for the samples treated with RNAi2 ($p = .05$, SEM = 0.11) relative to the DISC1 expression calculated in the Control RNAi condition.

3.3.2. Proteomic changes

Protein concentrations in individual samples were determined for the four conditions RNAi1, RNAi2, Control RNA and No RNAi and two-dimensional gel electrophoresis (2DPAGE) was performed in acrylamide gels. Gel analysis software 'Progenesis Samespots' was used to determine significant abnormal protein levels in different group (condition) comparisons; RNAi1 vs. Control RNAi, RNAi2 vs. Control RNAi, Control RNAi vs. NoRNAi, RNAi1 vs. RNAi2, RNAi1 vs. NoRNAi, and RNAi2 vs. NoRNAi. The No RNAi group was used to assess the effects of siRNA treatment itself on protein expression (off-target effects). Statistical analyses were conducted between the RNAi1 and RNAi2 and Control RNA.

Post alignment and analysis, the software identified a number of spots and generated a report with < 20 protein spots in each case comparison found to be of statistical significant ($p \leq .05$). Average fold change value and p-value (ANOVA) were used to compare common significant differences between RNAi1 vs. Control RNAi, and RNAi2 vs. Control RNAi. A Principal Component Analysis (PCA) performed with Progenesis SameSpots Statistical software on the groups showed a first component that accounted for 28.09% of the variance observed and a second component that accounted for 14.95% of the variance resulting in a clear distribution of the four groups (Figure 12).

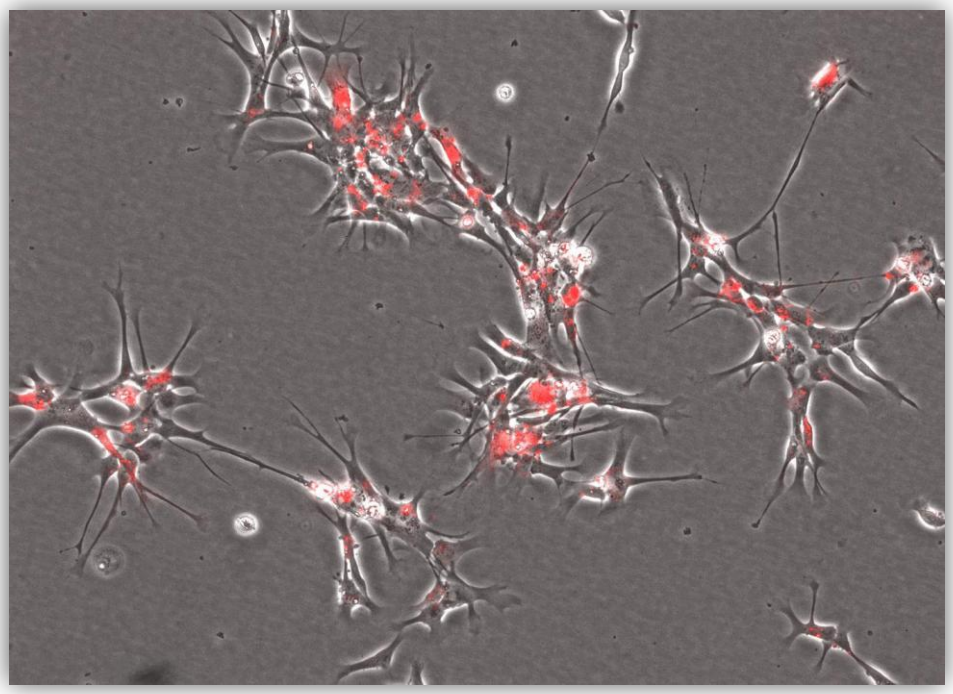

Figure 11. Image (x100) of CTXOE03 cells on day 3 of transfection with red fluorescent oligonucleotide

(Taken by Ms Kinsella)

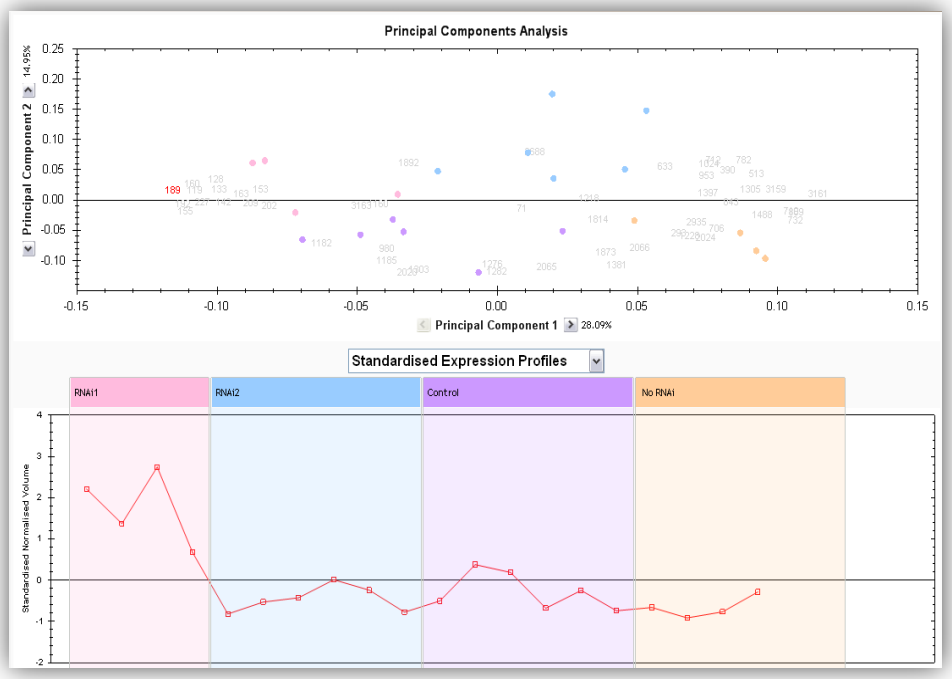

Figure 12. Principal Component Analysis (PCA) on the four groups.

RNAi1 (pink), RNAi2 (blue), Control (purple), NoRNAi (orange).

3.3.3. RNAi1 vs. Control RNAi

The comparison between the two groups yielded 8 spots with a statistically significant difference (Table 9). These spots were labelled as 1 (160), 2 (189), 3 (202) 4 (71), 5 (2020), 6 (2688), 7 (293) and 8 (1305) (Figure 13).

3.3.4. RNAi2 vs. Control RNAi

In the RNAi2 vs. Control RNAi condition comparison there were 18 protein spots of significance reported (Table 10). For the RNAi2 comparison these spots were labelled as 1 (1276), 2 (980), 3 (782) 4 (2020), 5 (2688), 6 (633), 7 (513), 8 (953), 9 (1282), 10 (712), 11 (1024), 12 (390), 13 (192), 14 (1303), 15 (1185), 16 (1381), 17 (1182) and 18 (2065) (Figure 14).

3.3.5. Common spots

There was a shared common significant change in two protein spots in both RNAi conditions compared with Control RNAi (Table 10). For the RNAi1 comparison these spots were labelled number 5 (Spot No 2020, p = .041, average fold change = .62) and number 6 (Spot No 2688, p = .044, average fold change = 1.6). For the RNAi2 comparison these spots were labelled number 4 (Spot No 2020, p = .004, average fold change = 0.5) and number 5 (Spot No 2688, p = .004, average fold change = 1.6).

On examination of the gel it can be determined that both spots on both gels lie on the bottom of the gel and slightly to the right implying that these proteins both have a low molecular weight and a pH at the top end of the pH 3-11 spectrum examined by the isoelectric focussing strip used in 2-D gel electrophoresis. These spots will from now on be referred to as Spot A (2020) and Spot B (2688) for clarity purposes.

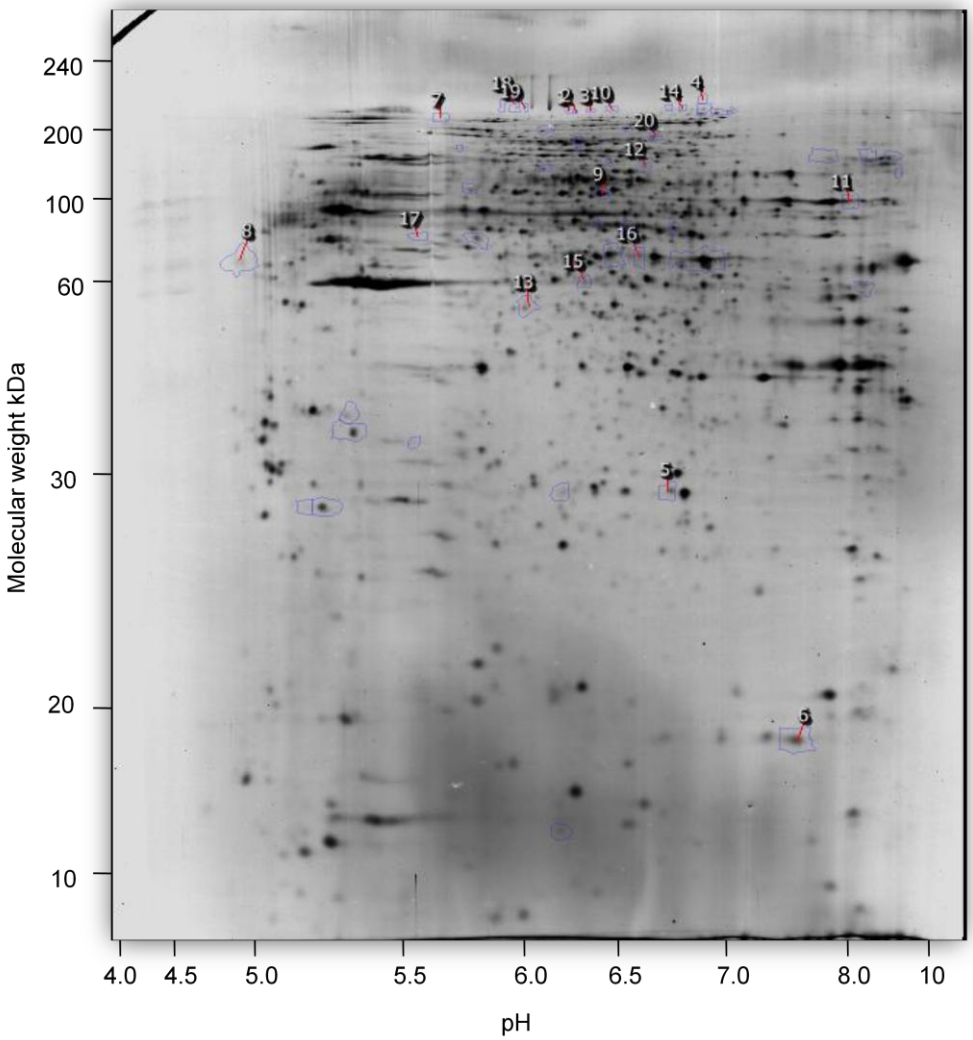

Figure 13. A representative image of 2-D gel of RNAi1 vs. Control RNAi comparison with the top 20 candidate spots identified and labelled.
Protein spots of significance in this case are labelled 5 and 6.

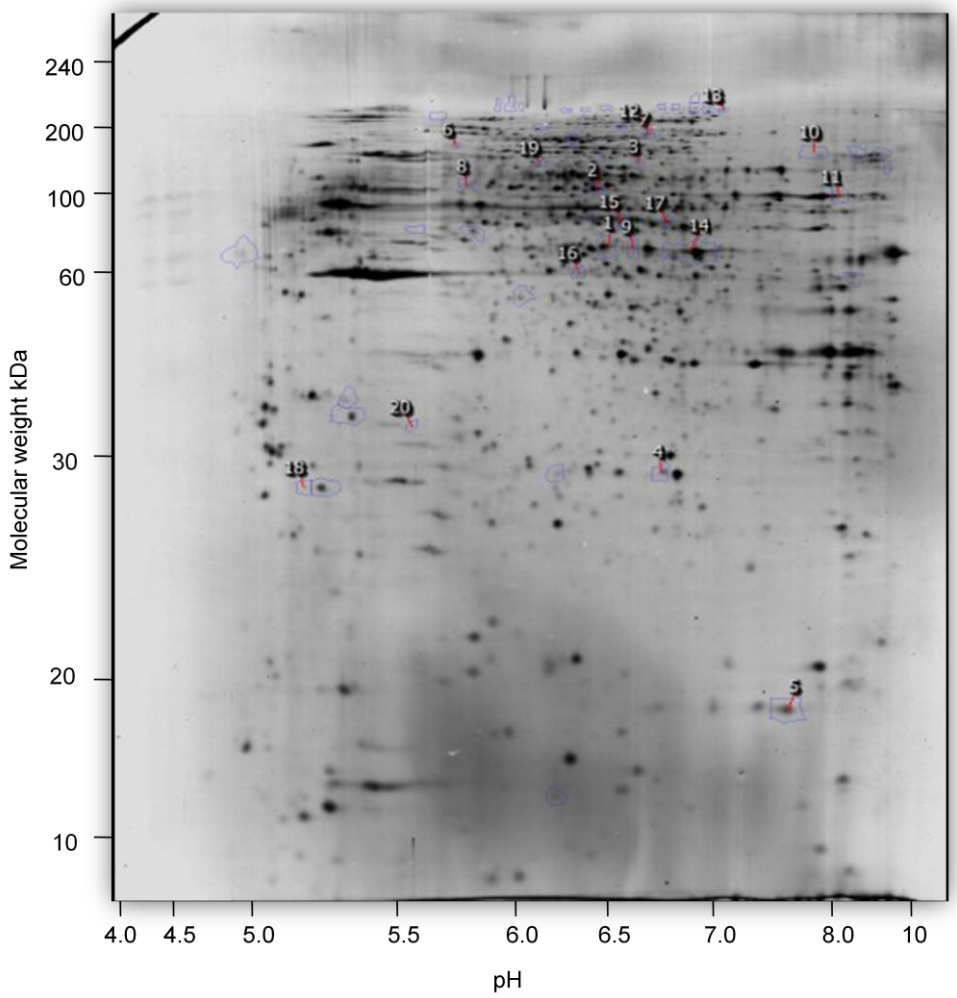

Figure 14. Representative Image of 2-D gel with top 20 candidate spots identified and labelled for RNAi2 vs. Control RNAi comparison.
Protein spots of significance in this case are labelled 4 and 5.

Table 9. Average normalised volumes and p values for the relevant spots.

Spot No	Anova (p)	Fold change	Spot label	ANV		Spot No	Anova (p)	Fold change	Spot label	ANV	
				RNAi1	Control					RNAi2	Control
160	.003	3.4	1	6.91E+04	2.03E+04	**1276**	.000	1.4	1	1.23E+06	1.74E+06
189	.004	3.4	2	4.70E+04	1.37E+04	**980^**	.000	1.4	2	7.44E+05	1.07E+06
202	.005	3.3	3	6.17E+04	1.87E+04	**782^**	.002	1.7	3	6.92E+04	4.00E+04
71	.016	1.8	4	7.53E+04	4.20E+04	**2020^***	.004	2	4	2.82E+05	5.65E+05
2020^*	.041	1.6	5	3.53E+05	5.65E+05	**2688^***	.004	1.6	5	2.79E+06	1.75E+06
2688^*	.044	1.6	6	2.80E+06	1.75E+06	**633**	.005	2.5	6	1.47E+05	5.83E+04
293	.044	1.5	7	1.58E+05	2.39E+05	**513^**	.012	1.8	7	1.73E+05	9.46E+04
1305	.048	2.1	8	5.60E+05	1.17E+06	**953**	.012	1.6	8	3.41E+05	2.07E+05
980^	.068	1.2	9	8.98E+05	1.07E+06	**712**	.014	1.7	10	7.32E+05	4.26E+05
153	.086	2	10	9.80E+04	4.86E+04	**1024^**	.014	1.8	11	9.56E+05	5.31E+05
1024^	.092	1.4	11	7.41E+05	5.31E+05	**1282^**	.014	2.2	9	2.69E+05	5.83E+05
782^	.124	1.3	12	5.28E+04	4.00E+04	**390**	.017	2.2	12	7.99E+04	3.61E+04
1488	.149	1.2	13	3.19E+05	2.61E+05	**192**	.018	1.7	13	2.65E+04	4.39E+04
155	.153	1.6	14	1.03E+05	6.50E+04	**1303**	.02	1.3	14	3.90E+06	5.23E+06
1381	.153	1.6	15	1.66E+05	2.64E+05	**1185**	.022	1.5	15	3.37E+05	5.08E+05
1282^	.165	1.6	16	3.57E+05	5.83E+05	**1381**	.031	1.8	16	1.48E+05	2.64E+05
1218	.187	1.7	17	1.05E+05	1.74E+05	**1182**	.043	1.4	17	1.91E+05	2.70E+05
133	.203	1.5	18	5.03E+05	3.41E+05	**2065**	.046	1.5	18	1.50E+05	2.33E+05
119	.214	1.5	19	1.55E+05	1.01E+05	**809**	.065	1.3	19	1.76E+05	1.39E+05
513^	.221	1.3	20	1.27E+05	9.46E+04	**1892**	.103	2.2	20	1.79E+05	8.04E+04

ANV = average normalised volumes. ^Common spots to both groups. * Significant spots common to both groups.

3.3.6. LC/MS/MS

Preparatory gels compatible with LC/MS/MS were prepared for each RNAi treatment group (Figure 15). A total of six gels per group with a load of 300 µg protein per gel were run in a single 12 gel batch. Spots A and B were excised on all 12 gels and taken to King's College Proteomics Facility for digestion and LC/MS/MS analysis.

The mass spectral data was processed into peak lists using ProteinLynx Global Server v2.2.5 with the following parameters: (1) MS survey – No background subtraction, SG smoothing 2 iterations 3 channels, peaks centroided (top 80%) no de-isotoping and (2) MS/MS – No background subtraction, SG smoothing 2 iterations 4 channels, peak centroiding (top 80%) no de-isotoping.

The peak lists was searched against the Swiss-Prot and a concatenated Swiss-Prot database using Mascot software v2.2 (http://www.matrixscience.com) using the following parameter specifications: precursor ion mass tolerance 1.2 Da, fragment ion mass tolerance 0.6 Da, tryptic digest with up to three missed cleavages, variable modifications: acetyl (protein N-term), carbamidomethylation (C), Gln->pyro-glu (N-term Q), oxidation (M) and phosphorylation (S, T and Y).

LC/MS/MS analysis and interrogation of the data against the Swiss-Prot database successfully identified proteins from the excised and digested 2D gel spots.

The results of the analysis and database searches are given in Table 10. Database generated files were uploaded into Scaffold 3 (v3_00_06) software (www.proteomesoftware.com) to create .sfd files (PR232 RSF_NB 2D gel spots 12112010). All samples were aligned in this software for easier interpretation and used to validate MS/MS based peptide assignments and protein identifications. Peptide assignments were accepted if they contained at least two unique peptide assignments and were established at 100% identification probability by the Protein Prophet algorithm (Nesvizhskii *et al.* 2003). The Threshold Identity Score corresponds to a 5% chance of incorrect assignment. Peptides identified below these probabilities were accepted following manual inspection of the raw data to ensure that fragment ions correctly match the assigned sequence.

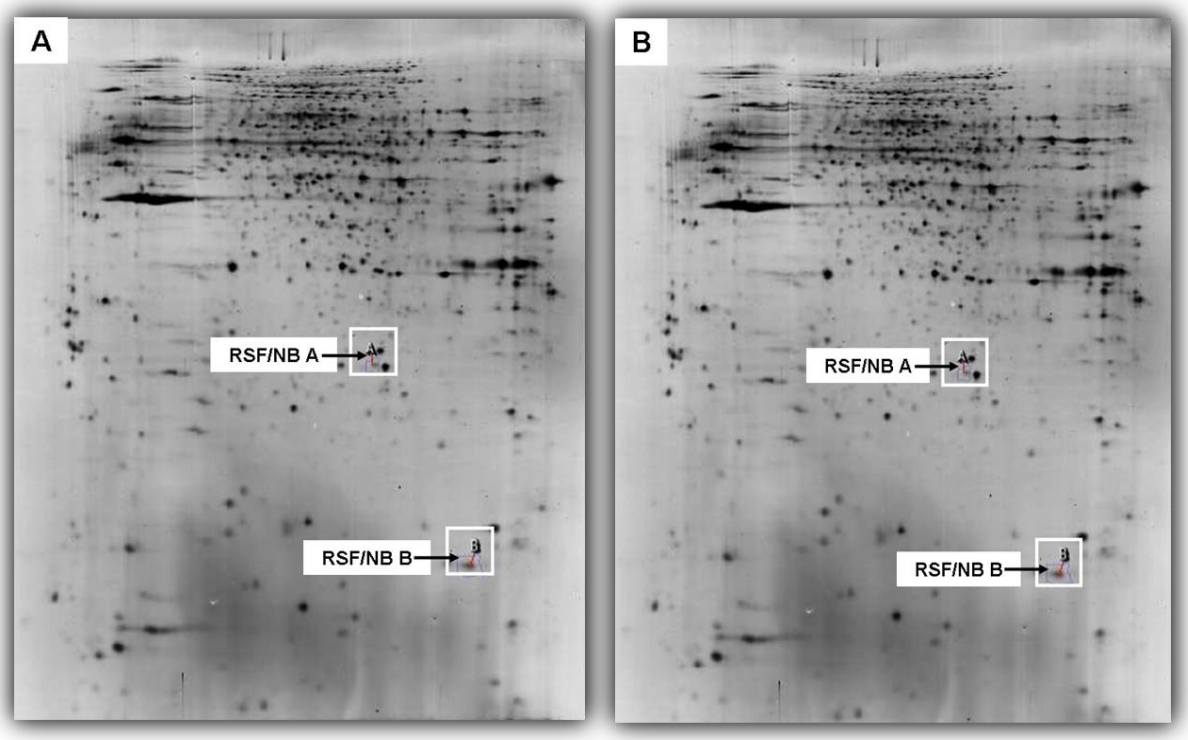

Figure 15. Protein spots common to both DISC1 RNAi1 and RNAi2 knockdowns.
Panel A represents 2D PAGE gel of RNAi1 vs. Control whilst Panel B represents a 2D PAGE gel of RNAi2 vs. Control. Labels highlight excised and pooled spots.

Table 10. Identities of the peptides identified (gene name, protein name).

Label Spot No.	Protein I.D.	Species	Accession No.	MW (Da)	pI
RSF/NB A (2020)	Dihydropteridine reductase(QDPR, DHPR)	Human	P09417	25773	6.9
RSF/NB B (2688)	Peptidyl-prolyl isomerase A (PPIA, Cyclophilin A)	Human	P62937	18001	7.7

3.4. Summary

In this study, the approach to the examination of the proteome in SCZ used is a novel discovery approach. It is based on the examination at the protein level of a mimicked haploinsufficiency of DISC1 in human neural progenitor cells using classical proteomic techniques. It was hypothesised that as the gene that is altered in the disorder was known (and although off target effects must also be considered); changes at the proteomic level could be directly related to the effects elicited by the knockdown of DISC1.

DISC1 was successfully knocked down in CTXOE03 cells via siRNA transfection, with expression reduced in both DISC1 RNAi treated groups (35% reduction in DISC1 expression for the samples treated with RNAi1 and a 38% reduction in DISC1 expression for the samples treated with RNAi2). Analysis of the cellular proteome was carried out by using 2DPAGE coupled to LC/MS/MS and results revealed significantly abnormal levels of several proteins across both DISC1 RNAi transfection groups.

Eight significant protein changes were found in the RNAi1 vs. Control RNAi comparison, and there were a total of 18 significant protein changes found in the RNAi2 vs. Control RNAi comparison. Analysis of both set of results yielded two spots which were identified in this study as being regulated by both RNAi groups with a statistically significant difference with the control RNAi group. These spots were then excised and processed through LC/MS/MS for identification. These analytes were dihydropteridine reductase (DHPR) and peptidyl-prolyl isomerase A (cyclophilin A). It is noteworthy to mention here that a single analyte per spot was obtained, suggesting that discovery approaches for knockout studies have great potential in neuropsychiatric disorders.

Dihydropteridine reductase (DHPR) maps to chromosome location 4p15.31 and is a protein present in cytoplasm, cytosol and mitochondrion that catalyzes the NADH-mediated reduction of quinonoid dihydrobiopterin. Dihydropteridine reductase deficiency presents as atypical phenylketonuria due to insufficient production of biopterin, a cofactor for phenylalanine hydroxylase. The product of this enzyme, tetrahydrobiopterin (BH-4), is an essential cofactor for phenylalanine, tyrosine, and tryptophan hydroxylases and is therefore important in the biosynthesis of biogenic amines like dopamine, norepinephrin and serotonin. The most obvious marker of DHPR deficiency, which leads to tetrahydrobiopterin or (BH4) deficiency is neonatal hyperphenilanemia. DHPR activity has been measured in blood in a small sample of people with SCZ and healthy controls but no differences were found between the groups (Szymanski *et al.* 1985). More recently, DHPR was found to be upregulated in the Wernicke's area (Martins-De-Souza *et al.* 2009a) and in the thalamus and CSF (Martins-De-Souza *et al.* 2010) of SCZ patients.

Cyclophilin A (PPIA) maps to chromosomic region 7p13 and is secreted from smooth muscle and macrophages in response to oxidative stress, which suggests a role in inflammation (Jin *et al.* 2004). In addition, PPIA catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and accelerate the folding of proteins. This protein is a cyclosporin binding-protein and may play a role in cyclosporin A-mediated immunosuppression. PPIA can also interact with several HIV proteins, including p55 gag, Vpr, and capsid protein, and has been shown to be necessary for the formation of infectious HIV virions. In neuropsychiatric disorders, the role of cyclophilin A is not yet fully explored. It appears that it has a role in apoptosis as it augments chromatinolysis synergically with apoptosis inducing factor (AIF) (Cande *et al.* 2004), which moves from mitochondria to nuclei following cerebral hypoxia-ischemia (Zhu *et al.* 2007). A recent study showed that it was downregulated in the anterior cingulate cortex of schizophrenic males (Martins-De-Souza *et al.* 2010) but was found in a different study by the same authors to be upregulated in the thalamus and CSF of SCZ patients (Martins-De-Souza *et al.* 2010) , which may suggest a region-specific profile expression. In addition, cyclophilin A appears to promote atherosclerosis in apolipoprotein-E deficient mice (Nigro *et al.* 2011), an intriguing relationship which may have clinical significance for dementia patients.

As a limitation of this study, significant down-regulation of dihydropteridine reductase and up-regulation of cyclophilin-A need to be confirmed by enzyme-linked immunosorbent assays (ELISAs) and work is currently being conducted in concjunction with Dr Bray to validate the finding. It is equally important to take into account that not all downstream translational changes may be noted at the time point of this experiment, and therefore many more proteomic changes may take place following DISC1 knockdown than have been reported here.

In summary, one of the strongest apparent risk factors for psychiatric illness is a (1;11)(q42;q14) chromosomal translocation disrupting the DISC1 gene, which co-segregates with psychotic and affective disorders in a large Scottish family. We have used RNA interference (RNAi) to model the reduction in DISC1 expression that is predicted to result from this translocation in cells derived from the human fetal cortex. 2-dimensional gel electrophoresis (2DE) and liquid chromatography–mass spectrometry (LC-MS) were used to identify proteins that were differentially expressed as a result of DISC1 knockdown in these cells. Two proteins, identified as dihydropteridine reductase and cyclophilin-A, showed significant expression changes that were shared by the two DISC1 RNAi conditions. Dihydropteridine reductase, encoded by the QDPR gene, recycles tetrahydrobiopterin (BH-4), an essential cofactor for tyrosine and tryptophan hydroxylases and therefore an important determinant of dopamine and serotonin synthesis. Cyclophilin-A, encoded by the PPIA gene,

has been implicated in a variety of functions, including protein folding and inflammation. Abnormal expression of these proteins could make a significant contribution to psychopathology in t(1;11)(q42;q14) carriers, and may be relevant to psychiatric disorders more generally.

CHAPTER 4: Examining the effects on brain and blood proteome of antipsychotic and antidepressant medication on F344 inbred rats

4.1. Rationale and aims

This study was performed to examine the effects of antidepressant and antipsychotic treatment on protein expression in mammalian brain tissue and plasma with the intention of identifying molecular pathways altered by the effect of antipsychotic medication. Due to obvious ethical and logistic constraints that limit access to human brain samples, the study was performed using adult inbred F344 rats in collaboration with Dr María Jesús Arranz and Dr Sridar Natesan (Section of Schizophrenia, Imaging and Therapeutics, Department of Psychosis Studies, Institute of Psychiatry, King's College London).

The hallmark symptom of SCZ, psychosis, appears to be susceptible to treatment with antipsychotic medication which, in the majority of cases, acts by blocking dopaminergic receptors. This is an apparently compensatory mechanism to excessive dopaminergic release from the presynaptic neuron, mainly in the striatum. Dopamine D2 blockade is directly correlated to treatment response (Kapur and Mamo, 2003) but by which mechanism these compounds exert their effects is far from being fully understood. It is known nonetheless, that antipsychotic treatment induces changes in gene expression, leading to RNA modifications (Chertkow *et al.* 2007; Thomas, 2006) that will eventually manifest in protein expression variations, potentially altering cell signalling and intracellular molecular pathways in a specific way.

With the underlying assumption that there are highly conserved signalling pathways like JAK/STAT, Notch, Wnt and Hedgehog that could be altered either by the disorder or by the action of these drugs, this study aimed to show that differential protein expression patterns induced by antipsychotic treatment could be explored using a classical proteomic approach. Moreover, the information obtained about modifiers of signalling pathways will be used for the interpretation of the results from the human studies carried out in this thesis.

In addition, comparison of protein expression profiling patterns between different types of drugs (antipsychotic and antidepressants, using a vehicle as control group) may identify individual expression profile pharmacological signatures which will shed light on unique mode of actions (in the case of clozapine) or on side-effect profiles.

A classic proteomic approach (2DPAGE/LC/MS/MS) was used in an animal model to identify expression changes on the proteome of mammalian plasma and striatum (a brain region associated with the pathophysiology of SCZ in humans) following chronic antipsychotic administration.

Thus, the main hypothesis tested in this study was that antipsychotic administration induces a pattern of protein expression unique to antipsychotic effect (i.e. not observed with

other treatments such as antidepressants) and is circumscribed to certain intra and extra-cellular pathways.

4.2. Material and methods

All experiments were conducted in compliance with the Animals Experimental Procedures Act (1986) and were approved by relevant local ethics committees.

4.2.1. Animals

Thirty-six adult male inbred F344 rats, weighing 225-250g on arrival, were housed in cages with a standard 12-hour light/dark cycle at 22^{o}C. Access to water and food consisting on a standard diet for rodents were provided *ad libitum*. Weight gain was monitored for the 14 days of treatment duration (Figure 16).

The environment including temperature, humidity, ventilation, lighting and noise was maintained according to standards set by the Home Office in the Code of Practice for the housing of animals used in scientific procedures (21/03/2005). Animals subjected to the same treatment (haloperidol, citalopram or control) were housed together in cages of four individuals each which resulted in three cages per treatment arm (each arm consisted of 12 animals). Animals were sacrificed in three consecutive days to minimise batch effects as follows: four 'test' animals and four controls were sacrificed on day one; 12 haloperidol-treated animals and 4 control animals on day two; and 12 citalopram-treated animals and four controls on day three.

In this study, the butyrophenone haloperidol, regarded mainly as a dopamine D2, D3 and D4 receptor blocking compound, was used to examine the downstream effects of antipsychotic medication in the proteome. The antidepressant citalopram, a selective serotonin reuptake inhibitor (SSRI), was used as a comparison to find the unique specific profile, specific to the antipsychotic action of haloperidol. In addition, a vehicle was also used as an inert compound to control for unspecific changes induced predominantly by the experimental design (Table 11).

The subcutaneous implantation of chronic continuous dosing Alzet® osmotic mini-pumps to deliver the selected treatment was performed by Dr Natesan according to the procedure described by Samaha et al (Samaha *et al.* 2007).

In brief, animals were anesthetised and minipumps containing vehicle (VEH; 0.5% glacial acetic acid/H_2O solution), haloperidol, or citalopram were implanted thorough a 1.5-cm-

wide incision in the animal's lower back and inserted between the scapulae. The dose of the antipsychotic was chosen based on D2/3 receptor occupancy level determined in Sprague Dawley rats to ensure that clinically appropriate levels were achieved (Natesan *et al.* 2008) while the dose of citalopram was adjusted according to the equivalent human dose as current practice in animal studies (Hesketh *et al.* 2007). Haloperidol was administered to the equivalent of 0.5 mg/kg/day and citalopram to 10 mg/Kg/day. Both agents were dissolved in the common solvent system which served both as vehicle and to treat the control group.

Table 11. Drugs used on animal study, target receptor and metabolic pathway.

Drug	Main effect	Metabolic pathway	Main receptor target	Other receptor targets
Haloperidol	Antipsychotic	CYP2D6 CYP3A4	D_2, D_3, D_4	Sigma (σ), (Antagonist) $5HT_2A$, α1, α2
Citalopram	Antidepressant	CYP3A4 CYP2C19	5-HTT	Sigma (σ) (Agonist), α1, H1, mAchR
Vehicle	N/A	N/A	N/A	N/A

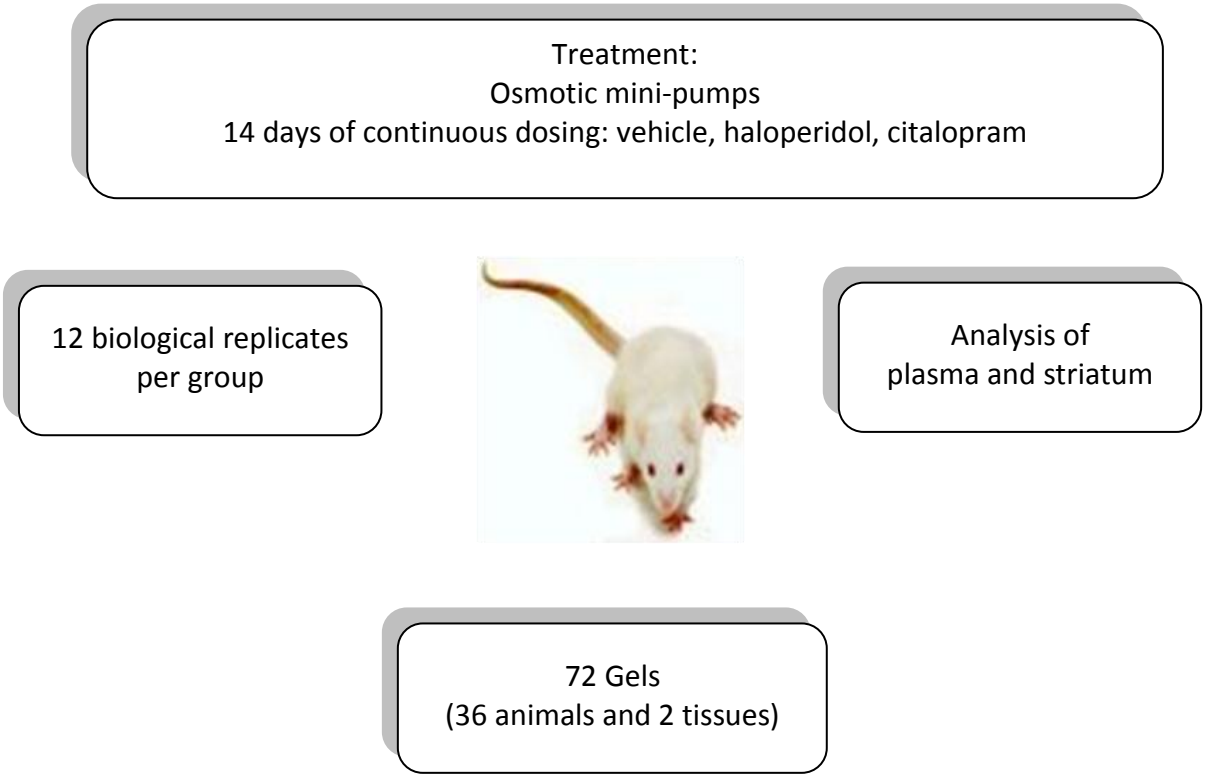

Figure 16. Experimental design of the animal study.

4.2.2. Protein analysis

Animal manipulations, sacrifice and relevant tissue dissections, excisions and extractions were performed by Dr Natesan. Animals were sacrificed by decapitation on day 14 of treatment. Brains were removed and the striatum area was dissected, washed on diethyl pyrocarbonate, snap frozen by placing them in isopentane over dry-ice and subsequently stored at -80^{o}C. Brain lysates were obtained using a standard lysis buffer. The solution was stirred for 10 minutes before being filtered. Following filtration, 1 g CHAPS, 0.5 g DTT and protease inhibitors were added to 48 ml of the solution and it was then aliquoted into 1 ml and stored at -80°C until use.

Brain tissue was homogenized at approximately 100mg/ml in lysis buffer using a manual tissue grinder. The homogenised sample was then transferred to a clean microtube and spun. The resulting precipitated tissue pellet was discarded and the supernatant was retained as the total lysate.

Truncal blood from sacrificed animals was collected in EDTA coated tubes immediately after decapitation using single-use sterile funnels and EDTA tubes. The tubes were placed at 4^{o}C for one hour and then spun at 3000 g for 8 minutes at 4^{o}C. Plasma was aliquoted in 0.5 ml eppendorf tubes and stored at -80^{o}C until needed for proteomic analyses.

Protein concentration of brain and plasma samples was determined according to the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard. Fifty micrograms of protein were used for the proteomic analysis of striatum and plasma samples.

In order to establish that the surgical procedure had successfully delivered the antipsychotic and antidepressant agents, it was decided to measure serotonin levels in brain and plasma as both agents are known to affect serotonergic transmission. We used an *ELISA Serotonin Assay* (Serotonin ELISA $^{\text{Fast Track}}$, Labor Diagnostika Nord) to measure serotonin levels in plasma and the *Serotonin Research ELISA* (Labor Diagnostica Nord) to measure the levels of serotonin in brain.

Plasma samples stored at -80°C were thawed on ice until they reached room temperature (RT) prior to being used. A volume of 25 µl per sample was used in the assay. Brain samples, which were preserved at -80°C were thawed on ice, eluted in lysis buffer and diluted 1:10 prior to use. A volume of 100 µl per sample was used.

Briefly, prepared lysates and plasma samples were defrosted and centifuged prior to use. Gels were run in three batches of 12 gels, comprising four samples per each group (vehicle, haloperidol and citalopram treated animals). The volume of sample determined to contain 50 µg of protein was dissolved in IPG rehydration buffer and 350 µl total volume was

loaded in 18cm Immobiline DryStrips (pH 3-11). Proteins were separated according to individual isoelectric point (PI) using the IPGphor™ (Amersham Bioscience) platform and subsequently equilibrated with dithiothreitol (DTT) and 2-Iodoacetamide.

The second dimension (separation of proteins by size) was performed using SDS-PAGE 2D acrylamide gels on an electrophoresis Ettan Dalt™ II (Amersham Biosciences) separation unit. Proteins were fixed to the gel matrix and stained with an optimised and adapted version of the protocol described by Hochstrasser *et al*. (Hochstrasser *et al.* 1988). Gels were scanned immediately after being developed and the subsequent image analysis was performed using Progenesis Samespots software (Nonlinear Dynamics, UK) version 3.2. Selected spots were excised from 2-DGE preparatory gels containing 300 µg of protein and gels were stained with modified Plus One silver stain kit (Amersham, UK). The spots were excised, suspended in double distilled water (ddH_2O) and taken to King's College London Proteomics Facility to be processed by Steve Lynham. The gel fragments were subjected to in-gel reduction, alkylation and digestion with trypsin prior to performing peptide mass fingerprinting and analysed by liquid chromatography tandem mass spectrometry (LC/MS/MS).

Image analysis was conducted using Progenesis SameSpots v3.3 software. Images were scanned using a Storm scanner and acquired as 12-bit .tiff files. Images were subjected to a process of quality control before conducting the analysis. One image per set (plasma, striatum and prefrontal) was selected as the reference image for the group. The reference images were: 26 (plasma), 2 (striatum) and 14 (prefrontal). The alignment was performed step-wise using built-in visual tools. An average of 50 vectors per image was added manually to specific protein patterns. Automatic vectors were subsequently added by the software using an image recognition algorithm and images were then 'aligned'. All the identified spots underwent an analysis process that included spot detection, background subtraction, normalisation and matching. The resulting spots were outlined and brought forward for the next stage of the image analysis. Individual images were grouped according to treatment arms (control, haloperidol, citalopram).

Different comparisons were performed (3 groups ANOVA and 2 groups non-parametric testing (haloperidol vs. control; citalopram vs. control and haloperidol vs. citalopram).Spots were statistically ordered according to p-value from the one way ANOVA and fold change. The statistical analysis was initially performed using the built-in Progenesis Stats software (a statistical resource containing Principal Component Analysis (PCA), Correlation Analysis, Power Analysis and False Discovery Rate and Q-values) and verification of the results obtained was subsequently conducted using SPSS 16.0.

4.3. Results

4.3.1. Serotonin ELISA

Levels in plasma were obtained for all subjects but brain levels were only ascertained for those subjects with enough tissue to run the assay without compromising the proteomic experiment (Table 12).

Brain serotonin levels, as predicted, were increased by citalopram and to a lesser extent by haloperidol. Plasma serotonin levels were increased in the haloperidol treated group but decreased following treatment with the SSRI (Figure 17). Surprisingly, the only non-significant difference in average serotonin levels was found between the increment due to haloperidol (47.5 pg/mg) and citalopram (77.6 pg/mg) in the brain of the treated animals (Table 13).

Thus, the ELISA results indicated that the drug reached the target tissues and confirmed that the implantation of the pump and the delivery of the drug were successfully achieved.

Table 12. Serotonin levels in plasma and brain tissue in F344 animals.

Group	Animal Number	ELISA plasma pg/µl	ELISA brain pg/mg
Control	1	229.8	
	2	436.1	
	3	418.9	19.9
	4	851.1	14.2
	5	497.5	25.3
	6	87.6	18.6
	7	333.1	38.7
	8	313.6	4.4
	9	683.6	
	10	555.7	
	11	563.4	6.2
	12	458.1	
	Mean	**452.4**	**18.2**
	SE	**58.7**	**4.43**
Haloperidol	13	540.1	52.4
	14	655.7	43.2
	15	437.0	53.1
	16	816.4	
	17	860.6	63.4
	18	447.3	48.0
	19	532.1	23.5
	20	675.9	72.7
	21	550.8	34.2
	22	462.9	58.8
	23	771.3	62.1
	24	475.5	11.3
	Mean	**602.1**	**47.5**
	SE	**43.3**	**5.53**
Citalopram	25	58.2	41.6
	26	50.9	
	27	41.1	18.0
	28	60.1	174.4
	29	46.4	74.6
	30	53.6	
	31	95.7	27.5
	32	65.6	
	33	74.8	50.5
	34	37.1	105.3
	35	66.4	111.4
	36	59.0	95.0
	Mean	**59.1**	**77.6**
	SE	**4.57**	**16.5**

Table 13. Student's t-test (two-tailed) results for serotonin levels in plasma and brain tissue in F344 animals.

	ELISA PLASMA t-test (p)	ELISA BRAIN t-test (p)
Control vs. Haloperidol	0.017	0.001
Control vs. Citalopram	3.50E-05	0.007
Haloperidol vs. Citalopram	7.28E-08	0.116

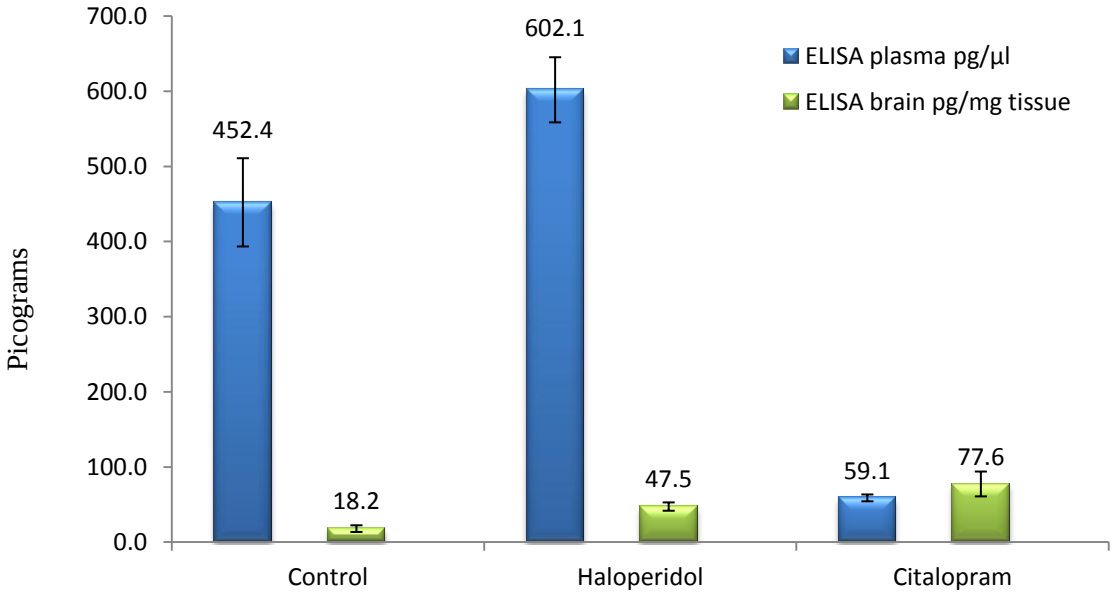

Figure 17. Mean serotonin levels in plasma and brain of F344 animals following treatment with haloperidol and citalopram.

4.3.2. Plasma

Thirty six images, 12 per group, were selected for image analysis. The image corresponding to animal number 26 from the citalopram treated group was selected as the reference image against which the remaining 35 were aligned. Alignment of the images yielded 1724 theoretical potential spots which were subsequently manually curated, leaving 347 spots with an Anova $p < .05$ as calculated by Progenesis Stats. Out of those, 96 of them were deemed to be actual spots rather than gel artefacts (Figure 18).

Using these 96 spots, a Principal Component Analysis (PCA) was conducted and a clear group separation between the haloperidol treated, citalopram treated and vehicle treated groups was observed in the sample (Figure 19) indicating that using the combined information of protein expression in these selected spots, gels were correctly assigned to the treatment group they belonged to with very little overlap or outliers.

To validate these results, an analysis of variance (ANOVA) was conducted to test for differences between the means of each spot of the three different groups in our study using SPSS 16.0 for windows. Spots that had a FDR (False Discovery Rate) < 3% and that could be unmistakably identified and excised from the gels were selected (Table 14).

On a confirmatory statistical analysis, the values of the haloperidol and citalopram groups were compared individually as independent samples with the control group using a non-parametric test (Mann-Whitney U test) (Table 15 and 16). Accordingly, the following 19 spots were selected from the list and sent to MS for ID: 699, 694, 668, 711, 708, 943, 661, 946, 1669, 763, 985, 947, 1126, 1679, 1681, 1127, 862, 837 and 240 (Table 17).

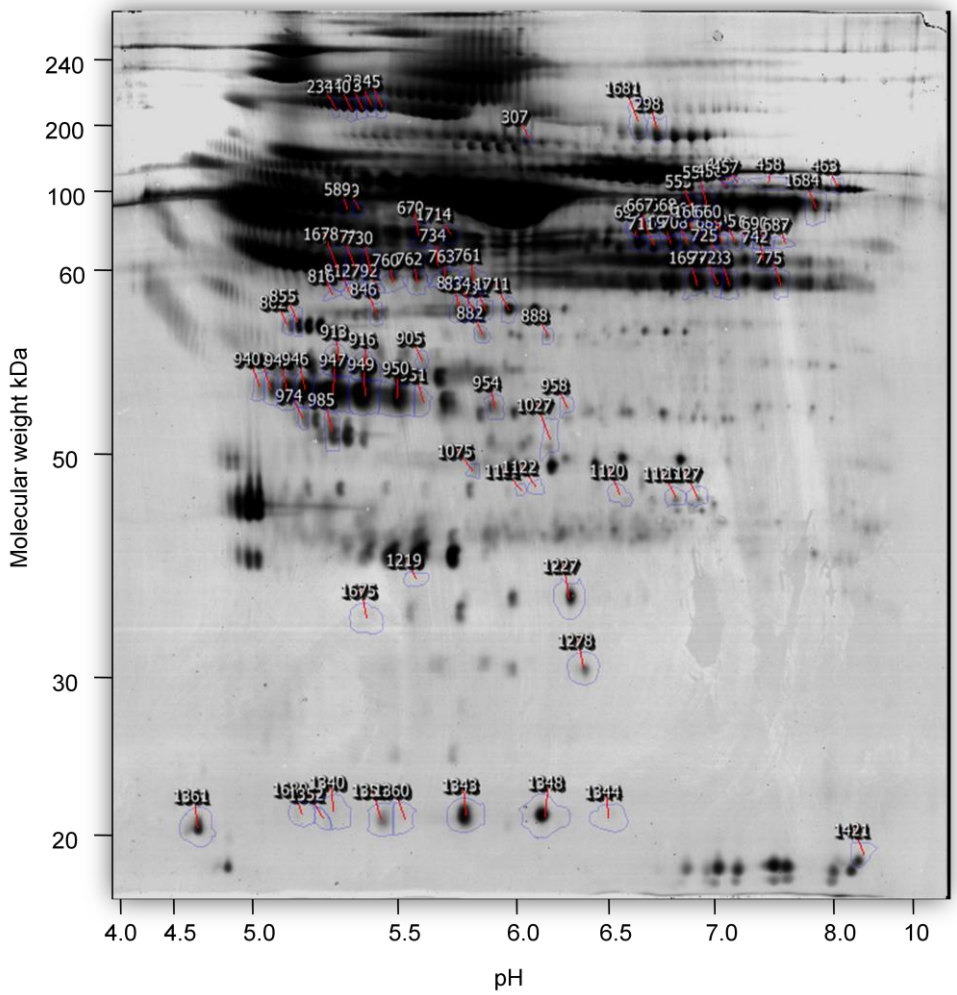

Figure 18. Image of rat plasma proteome corresponding to animal 26 with all the spots included in subsequent statistical analysis.

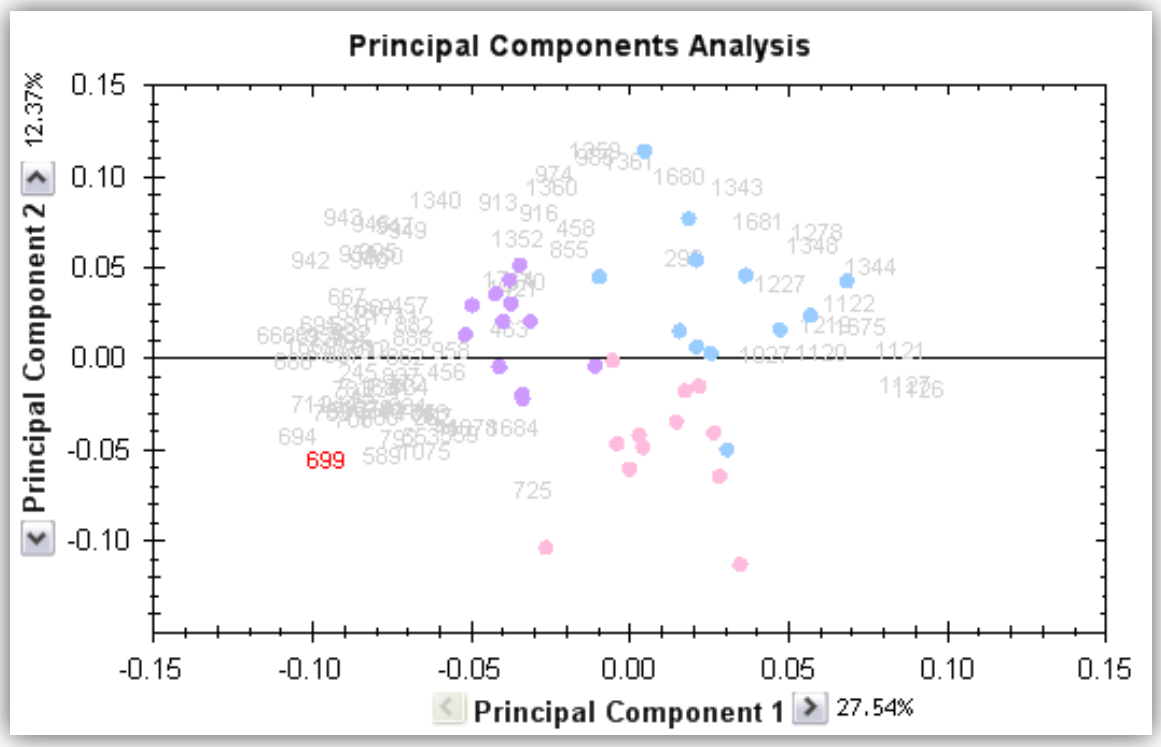

Figure 19. PCA of 96 significant plasma spots in rats included in analysis.
Principal component 1 (27.55% of total variance). Principal component 2 (12.35% of total variance). Groups: Control (pink), haloperidol (blue), citalopram (purple). Number 699 is the spot which was given the smallest p value in the ANOVA.

Table 14. Summary of top plasma spots in rats ranked as per ANOVA results between the three treatment groups in SPSS.

Spot No.	Chi-Square	df	Asymp. Sig.	Ranked	Theor Prob	FDR	%
694	19.676	2	0.000	3	0.011	0.005	0.498
668	19.383	2	0.000	4	0.014	0.004	0.433
708	19.059	2	0.000	5	0.018	0.004	0.407
699	18.817	2	0.000	6	0.021	0.004	0.383
711	18.587	2	0.000	7	0.025	0.004	0.368
661	18.434	2	0.000	8	0.029	0.003	0.348
946	17.416	2	0.000	10	0.036	0.005	0.463
947	17.321	2	0.000	12	0.043	0.004	0.404
943	16.029	2	0.000	14	0.050	0.007	0.661
1669	15.362	2	0.000	16	0.057	0.008	0.808
763	13.713	2	0.001	22	0.079	0.013	1.340
985	13.383	2	0.001	24	0.086	0.014	1.448
1681	11.815	2	0.003	38	0.136	0.020	2.003
1126	11.596	2	0.003	40	0.143	0.021	2.123
862	11.497	2	0.003	42	0.150	0.021	2.125
837	10.977	2	0.004	46	0.164	0.025	2.516
1679	10.857	2	0.004	48	0.171	0.026	2.560
1127	10.815	2	0.004	49	0.175	0.026	2.561
240	10.378	2	0.006	53	0.189	0.029	2.946

These spots were present in the ANOVA list from Progenesis SameSpots and could be identified and excised from the gel.

Table 15. Summary of top plasma spots in rats ranked as per Mann-Whitney results in SPSS between the haloperidol and the control treated.

Spot No.	Mann-Whitney Progenesis (p)	Fold change	Mann-Whitney SPSS (p)	SPSS Rank	Label on SameSpots	ANV control	ANV haloperidol
699	0.000	2	0.001	4	p1	2.91E+06	1.45E+06
694	0.002	1.8	0.004	10	p2	2.60E+06	1.48E+06
708	0.006	1.7	0.008	14	p5	1.41E+06	8.46E+05
763	0.008	1.7	0.009	20	P10	2.29E+06	1.36E+06
1679	0.019	1.5	0.015	30	P14	5.65E+06	3.89E+06
711	0.017	1.5	0.043	39	P4	1.44E+06	9.79E+05
943	0.052	1.6	0.057	45	P6	1.57E+06	2.48E+06
946	0.070	1.6	0.106	71	p8	2.83E+06	4.41E+06
661	0.141	1.3	0.133	84	p7	1.35E+06	1.04E+06
1669	0.108	1.5	0.149	98	P9	3.26E+06	2.24E+06
668	0.189	1.3	0.166	103	P3	1.66E+06	1.29E+06

These spots were present in the ANOVA list from Progenesis SameSpots and could be identified and excised from the gel. AVN = Average Normalised Volume.

Table 16. Summary of top spots ranked as per Mann-Whitney results in SPSS between the citalopram and the control treated group.

Spot No.	Mann-Whitney Progenesis (p)	Fold change	Mann-Whitney SPSS (p)	SPSS Rank	Label on SameSpots	ANV control	ANV citalopram
946	1.35E-04	2.6	0.000	3	p8	2.83E+06	7.29E+06
943	2.71E-05	3.2	0.000	5	P6	1.57E+06	4.99E+06
668	2.42E-04	1.8	0.001	10	P3	1.66E+06	2.96E+06
1127	3.01E-04	1.6	0.001	11	P16	1.46E+06	8.93E+05
661	2.89E-04	1.8	0.001	13	P7	1.35E+06	2.46E+06
1126	0.001	1.7	0.003	24	P13	8.84E+05	5.16E+05
240	0.018	1.7	0.004	26	P19	1.20E+06	1.99E+06
862	0.017	1.9	0.007	38	P17	3.63E+05	5.61E+05
711	0.003	1.4	0.004	30	P4	1.44E+06	2.03E+06
1669	0.004	1.6	0.008	41	P9	3.26E+06	5.38E+06
708	0.017	1.3	0.024	62	p5	1.41E+06	1.85E+06
694	0.004	1.4	0.028	67	p2	2.60E+06	3.62E+06

These spots were present in the ANOVA list from Progenesis SameSpots and could be identified and excised from the gel. AVN = Average Normalised Volume.

Table 17. Plasma spots selected for MS with average normalised volumes.

Spot No.	Mann-Whitney Progenesis (p)	Fold	Label	Control	Haloperidol	Citalopram
699	5.71E-07	2.5	p1	2.91E+06	1.45E+06	3.64E+06
694	6.92E-07	2.5	p2	2.60E+06	1.48E+06	3.62E+06
668	5.76E-06	2.3	P3	1.66E+06	1.29E+06	2.96E+06
711	6.04E-06	2.1	P4	1.44E+06	9.79E+05	2.03E+06
708	1.48E-05	2.2	P5	1.41E+06	8.46E+05	1.85E+06
943	2.93E-05	3.2	P6	1.57E+06	2.48E+06	4.99E+06
661	1.06E-04	2.4	p7	1.35E+06	1.04E+06	2.46E+06
946	2.09E-04	2.6	p8	2.83E+06	4.41E+06	7.29E+06
1669	3.30E-04	2.4	P9	3.26E+06	2.24E+06	5.38E+06
763	3.63E-04	2.3	P10	2.29E+06	1.36E+06	3.10E+06
985	6.35E-04	1.5	P11	3.18E+06	4.78E+06	4.62E+06
947	9.72E-04	2.1	P12	9.80E+06	1.40E+07	2.02E+07
1126	.003	2.2	P13	8.84E+05	1.12E+06	5.16E+05
1679	.003	1.5	P14	5.65E+06	3.89E+06	5.79E+06
1681	.003	1.8	P15	2.13E+06	3.81E+06	2.48E+06
1127	.005	2	P16	1.46E+06	1.80E+06	8.93E+05
862	.006	1.9	P17	3.63E+05	2.91E+05	5.61E+05
837	.011	1.5	P18	2.30E+06	1.85E+06	2.83E+06
240	.018	1.7	P19	1.20E+06	1.15E+06	1.99E+06

The location of the spots is shown in Figure 20. Spots p1, p2, p3, p4, p5, p7 and p9 clustered together and were likely to correspond to isoforms of the same protein(s). Similarly, p6, p8 and p12 formed another distinct group, as well as p13 and p16.

These spots were excised from the gel and taken for identification to the King's College Proteomics Facility. The results obtained from the LC/MS/MS procedure are summarised in tables 30 and 31. The top hits for spots p1, p2, p3, p4, p5, p7 and p9 were fibrinogen α and beta chain in all of them, making these analytes highly likely to be responsible for the overall expression changes observed in this group. The normalised average volumes of spots p1, p2, p3 and p7 were increased in the citalopram treated animals and decreased in the haloperidol group compared to controls, whereas the expression of p4 and p5 was increased in the citalopram group but highly increased in the haloperidol treated animals.

The other 'group' of proteins likely to be isoforms of the same species was formed by p6, p8 and p12 which yielded α-1-macroglobulin as their top hit. The expression of p6 and p8 was increased in the citalopram treated group whereas p12 was raised in the control group.

Spots p13 (decreased in haloperidol treated animals) and p16 (increased in citalopram treated animals) contained carbonic anhydrase 1 and 2, Ig kappa chain C region, A allele and Ig lambda-2 chain C-region.

Spot p10 (decreased in haloperidol treated animals and increased in the citalopram group) contained fibrinogen gamma chain, complement C3 and fibrinogen beta chain; p11 (decreased in controls), haptoglobin and anionic trypsin-2, p14 (decreased in haloperidol treated animals), α-1-antiproteinase, angiotensin, fibrinogen gamma chain and Vitamin D-binding protein; p15 (increased in haloperidol treated animals), plasminogen and serotransferrin; p17 (increased in citalopram treated animals), apolipoprotein A-IV and ATP synthase subunit beta, mitochondrial; and p18 and p19 (both increased in citalopram treated animals), complement C3.

Summarising, haloperidol increased the expression of isoforms of fibrinogen alpha and beta chain (p4, p5), isoforms of α-1-macroglobulin (p6, p8), haptoglobin and anionic trypsin-2 (p11), plasminogen and serotransferrin (p15); and decreased the expression of isoforms of fibrinogen alpha and beta chain (p1, p2, p3, p7, p9), fibrinogen gamma chain (p10), an isoform of α-1-macroglobulin (p12), carbonic anhydrase 1 and 2, Ig kappa chain C region, A allele and Ig lambda-2 chain C-region (p13), α-1-antiproteinase, angiotensin, fibrinogen gamma chain and vitamin D-binding protein (p14), apolipoprotein A-IV and ATP synthase subunit beta, mitochondrial (p17) and complement C3 (p18). Citalopram increased the expression of isoforms of fibrinogen alpha and beta chain (p1, p2, p3, p4, p5, p7, p9), an isoform of α-1-

macroglobulin (p6, p8), fibrinogen gamma chain (p10), haptoglobin and anionic trypsin-2 (p11), carbonic anhydrase 1 and 2, Ig kappa chain C region, A allele and Ig lambda-2 chain C-region (p16), apolipoprotein A-IV and ATP synthase subunit beta, mitochondrial (p17) and complement C3 (p18). Citalopram decreased the expression of an isoform of α-1-macroglobulin (p12), carbonic anhydrase 1 and 2, Ig Kappa chain C region, A allele and Ig lambda-2 chain C-region (p13) (Table 18).

The direction of the expression of the selected spots is summarised in Table 19.

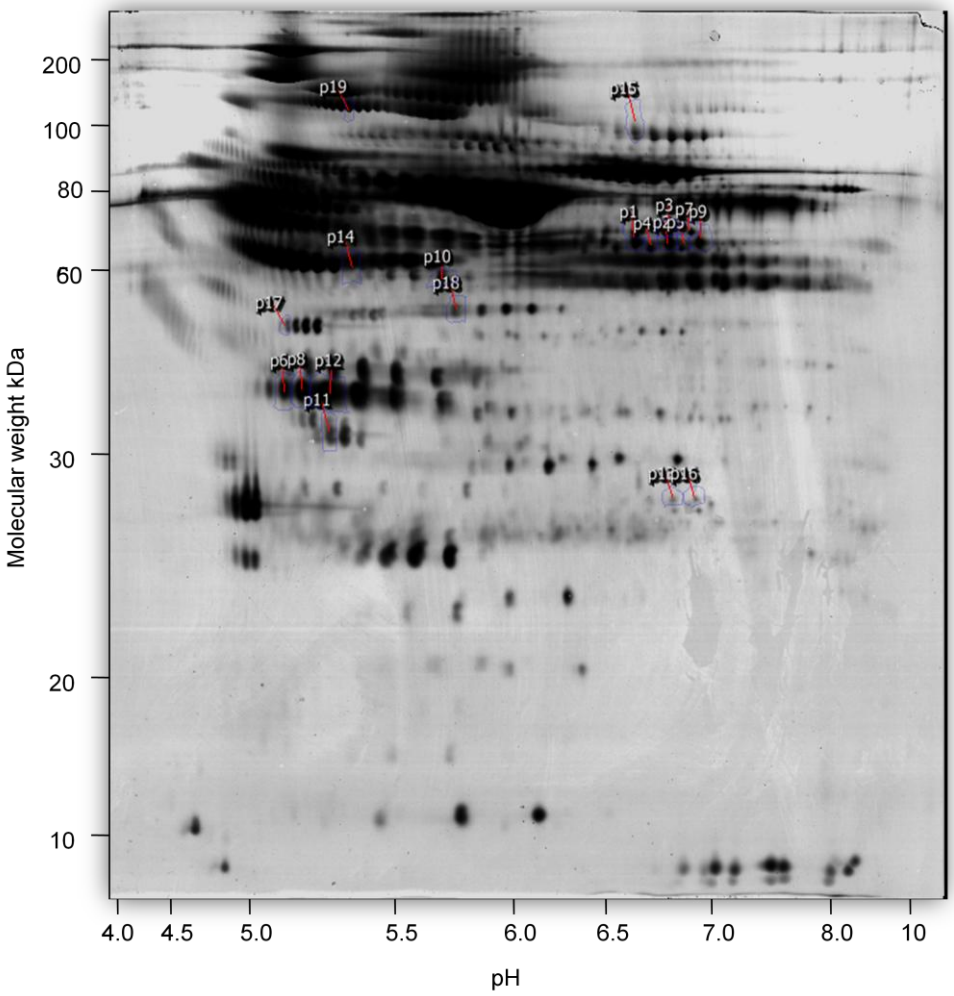

Figure 20. Plasma Spots selected for LC/MS/MS.

Table 18. MS results from the selected plasma spots in the animal study.

Spot label (number)	Protein I.D.	Accession No.	MW (Da)	pI	No. Peptides Matched	No. Unique Peptides	Percentage Coverage
P1 (699)	Fibrinogen alpha chain	P06399	86632	5.5	44	21	29%
	Fibrinogen beta chain	P14480	54201	7.9	29	15	37%
	Beta-2-glycoprotein 1	P26644	33175	8.6	6	4	16%
	Ig gamma-2B chain C region	P20761	36474	7.7	3	3	17%
	Serotransferrin	P12346	76346	7.1	1	1	2%
P2 (694)	Fibrinogen alpha chain	P06399	86632	5.5	41	20	29%
	Fibrinogen beta chain	P14480	54201	7.9	33	19	40%
	Beta-2-glycoprotein 1	P26644	33175	8.6	2	2	8%
	Ig gamma-2B chain C region	P20761	36474	7.7	2	1	6%
P3 (668)	Fibrinogen beta chain	P14480	54201	7.9	56	27	56%
	Fibrinogen alpha chain	P06399	86632	5.5	24	15	25%
	Beta-2-glycoprotein 1	P26644	33175	8.6	5	4	11%
	Ig gamma-2B chain C region	P20761	36474	7.7	1	1	5%
	Anionic trypsin-2	P00763	26211	4.8	1	1	4%
P4 (711)	Fibrinogen alpha chain	P06399	86632	5.5	8	5	9%
	Fibrinogen beta chain	P14480	54201	7.9	4	3	10%
	Ig gamma-2B chain C region	P20761	36474	7.7	4	2	11%
P5(708)	Fibrinogen beta chain	P14480	54201	7.9	49	24	55%
	Fibrinogen alpha chain	P06399	86632	5.5	21	13	21%
	Ig gamma-2B chain C region	P20761	36474	7.7	7	5	24%
	Beta-2-glycoprotein 1	P26644	33175	8.6	1	1	5%
P6 (943)	Alpha-1-macroglobulin	Q63041	2E+05	6.5	7	7	6%
P7 (661)	Fibrinogen alpha chain	P06399	86632	5.5	30	19	29%
	Pyruvate kinase isozymes M1/M2	P11980	57781	6.6	5	3	8%
P8 (946)	Alpha-1-macroglobulin	Q63041	2E+05	6.5	8	7	5%
P9 (1669)	Fibrinogen beta chain	P14480	54201	7.9	56	24	54%
P10 (763)	Fibrinogen gamma chain	P02680	50600	5.6	36	19	60%
	Complement C3	P01026	2E+05	6.1	2	1	2%
	Fibrinogen beta chain	P14480	54201	7.9	2	1	3%
P11 (985)	Haptoglobin	P06866	38539	6.1	14	10	25%
	Anionic trypsin-2	P00763	26211	4.8	1	1	4%
P12 (947)	Alpha-1-macroglobulin	Q63041	2E+05	6.5	15	11	8%
	Tubulin alpha-1C chain	Q6AYZ1	49905	5.0	2	1	3%
P13 (1126)	Carbonic anhydrase 2	P27139	29096	6.9	19	2	9%
	Ig kappa chain C region, A allele	P01836	11725	5.0	6	2	25%
	Ig lambda-2 chain C region	P20767	11311	5.8	4	2	32%
	Carbonic anhydrase 1	B0BNN3	28282	6.9	3	1	4%
P14 (1679)	Alpha-1-antiproteinase	P17475	46107	5.7	33	8	27%
	Angiotensinogen	P01015	51949	5.4	5	3	9%
	Fibrinogen gamma chain	P02680	50600	5.6	13	2	7%
	Vitamin D-binding protein	P04276	53509	5.7	9	1	4%
P15 (1681)	Plasminogen	Q01177	90477	6.8	19	13	16%
	Serotransferrin	P12346	76346	7.1	2	1	2%
P16 (1127)	Carbonic anhydrase 2	P27139	29096	6.9	17	3	20%
	Carbonic anhydrase 1	B0BNN3	28282	6.9	6	2	11%
	Ig kappa chain C region, A allele	P01836	11725	5.0	4	2	25%
	Ig lambda-2 chain C region	P20767	11311	5.8	2	2	32%
P17 (862)	Apolipoprotein A-IV	P02651	44429	5.1	81	16	51%
	ATP synthase subunit beta, mitochondrial	P10719	56318	5.2	6	2	5%
P18 (837)	Complement C3	P01026	186342	6.1	31	3	2%
P19 (240)	Complement C3	P01026	186342	6.1	9	3	3%

Table 19. Summary of direccion of expression from the selected plasma spots.

Spot label (number)	Protein I.D.	Haloperidol	Citalopram
P1 (699)	Fibrinogen alpha chain Fibrinogen beta chain Beta-2-glycoprotein 1 Ig gamma-2B chain C region Serotransferrin	↓	↑
P2 (694)	Fibrinogen alpha chain Fibrinogen beta chain Beta-2-glycoprotein 1 Ig gamma-2B chain C region	↓	↑
P3 (668)	Fibrinogen beta chain Fibrinogen alpha chain Beta-2-glycoprotein 1 Ig gamma-2B chain C region Anionic trypsin-2	↓	↑
P4 (711)	Fibrinogen alpha chain Fibrinogen beta chain Ig gamma-2B chain C region	↑	↑
P5 (708)	Fibrinogen beta chain Fibrinogen alpha chain Ig gamma-2B chain C region Beta-2-glycoprotein 1	↓	↑
P6 (943)	Alpha-1-macroglobulin	↑	↑
P7 (661)	Fibrinogen alpha chain Pyruvate kinase isozymes M1/M2	↓	↑
P8 (946)	Alpha-1-macroglobulin	↑	↑
P9 (1669)	Fibrinogen beta chain	↓	↑
P10 (763)	Fibrinogen gamma chain Complement C3 Fibrinogen beta chain	↓	↑
P11 (985)	Haptoglobin Anionic trypsin-2	↑	↑
P12 (947)	Alpha-1-macroglobulin Tubulin alpha-1C chain	↓	↓
P13 (1126)	Carbonic anhydrase 2 Ig kappa chain C region, A allele Ig lambda-2 chain C region Carbonic anhydrase 1	↓	↓
P14 (1679)	Alpha-1-antiproteinase Angiotensinogen Fibrinogen gamma chain Vitamin D-binding protein	↓	~
P15 (1681)	Plasminogen Serotransferrin	↑	~
P16 (1127)	Carbonic anhydrase 2 Carbonic anhydrase 1 Ig kappa chain C region, A allele Ig lambda-2 chain C region	~	↑
P17 (862)	Apolipoprotein A-IV ATP synthase subunit beta, mitochondrial	↓	↑
P18 (837)	Complement C3	↓	↑
P19 (240)	Complement C3	↑	~

4.3.3. Striatum

In a separate set of experiments, thirty six images, 12 per treatment group were selected for image analysis. The image corresponding to animal number 2 (Figure 21), from the control group was chosen as the reference image against which the remaining 35 were aligned. Alignment of the images yielded 1491 theoretical potential spots which were subsequently manually curated, leaving 234 spots with an Anova $p < .05$ as calculated by the program's statistical package. Out of those, 106 were deemed to be actual spots rather than gel artefacts.

Similarly to the analysis in the plasma samples, a PCA was conducted (Figure 22) using these 106 spots. Although the three groups are clearly visible in the graphic representation of the PCA, the separation is less clear than in plasma.

SPSS 16.0 was used in parallel with the raw data on average normalised volumes for each one of the spots exported to calculate the ANOVA and to conduct non-parametric tests to compare the control group firstly to the haloperidol and then to the citalopram one.

The ANOVA results are detailed in Appendix 2 and a summary of the values for the spots that were successfully identified and excised from the preparatory gels are given in Table 32. A summary of the results from non-parametric tests are shown in tables 33 and 34, below. Accordingly, the following 18 spots were selected from the list and sent to MS for ID: 877, 1159, 1228, 445, 729, 1039, 1163, 1143, 963, 1113, 595, 968, 1142, 838, 961, 1214, 908 and 642 (Table 20).

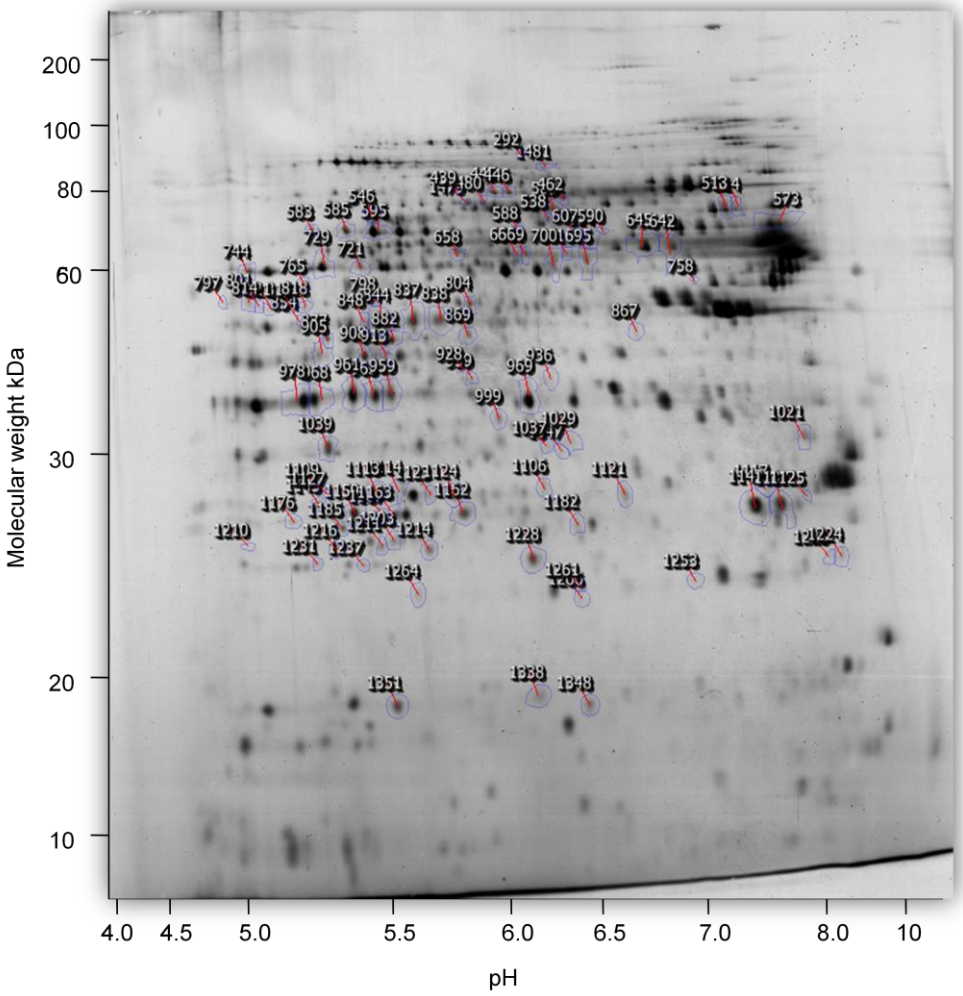

Figure 21. Image corresponding to animal 2 with the 106 significant spots included in analysis after manual curation of artefacts marked.

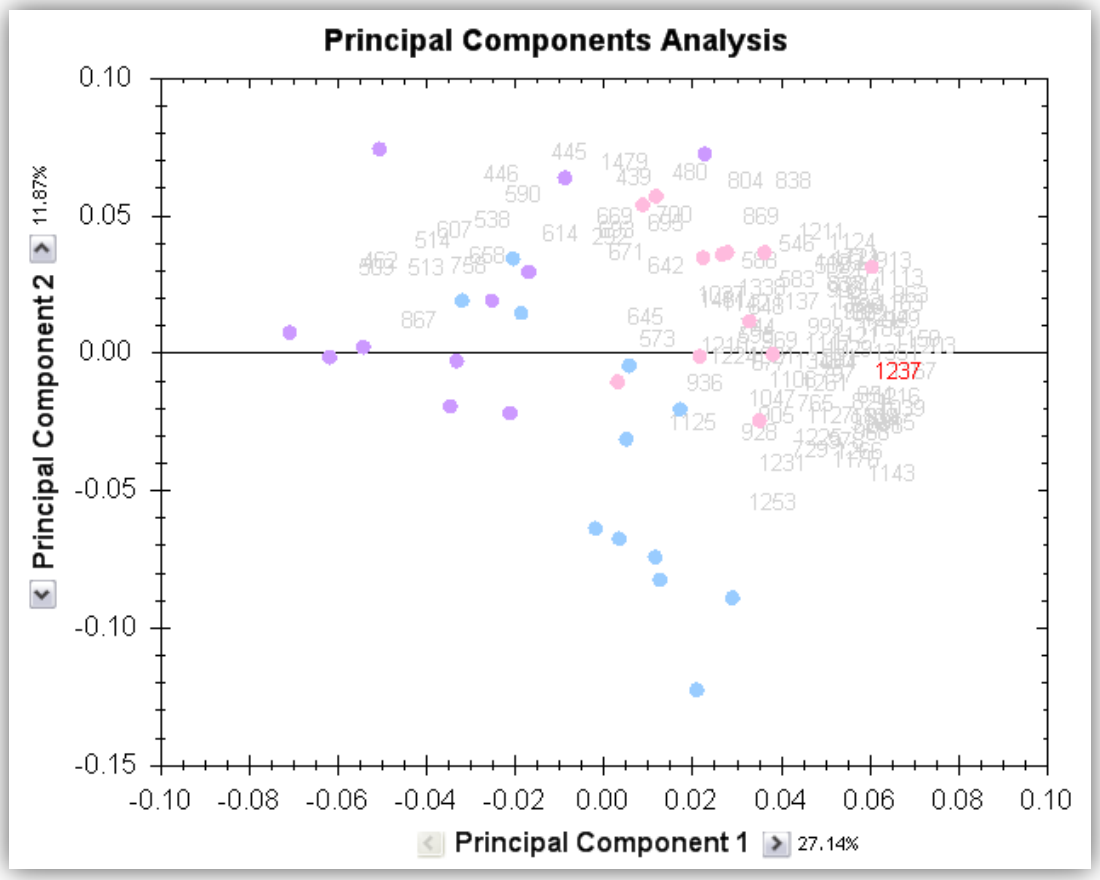

Figure 22. PCA of 96 significant spots included in analysis.
Principal Component 1 (27.14% of total variance). Principal Compoenent 2 (11.87% of total variance). Control (pink), haloperidol (blue), citalopram (purple). 1237 is the spots which was given the smallest p value in the ANOVA.

Table 20. Summary of top striatum spots in rats ranked as per ANOVA results between the three treatment groups in SPSS.

Spot no.	Chi-Square	df	p	Rank	Theor Prob	FDR	%
877	13.855	2	0.001	2	0.003	0.300	29.955
1159	12.845	2	0.002	5	0.008	0.198	19.848
968	11.656	2	0.003	10	0.016	0.180	17.986
1113	11.480	2	0.003	13	0.021	0.151	15.107
1228	10.909	2	0.004	19	0.031	0.138	13.756
1143	10.351	2	0.006	23	0.038	0.150	15.020
1163	10.205	2	0.006	24	0.039	0.155	15.482
595	9.769	2	0.008	29	0.047	0.159	15.932
729	9.416	2	0.009	32	0.052	0.172	17.230
445	9.380	2	0.009	33	0.054	0.170	17.006
1039	8.757	2	0.013	42	0.069	0.183	18.252
838	8.309	2	0.016	47	0.077	0.204	20.398
963	7.984	2	0.018	53	0.087	0.213	21.281
908	7.368	2	0.025	68	0.111	0.226	22.575
961	7.225	2	0.027	70	0.115	0.236	23.550
1142	7.100	2	0.029	72	0.118	0.244	24.375
1214	7.100	2	0.029	73	0.119	0.240	24.041
642	6.903	2	0.032	77	0.126	0.252	25.150

These spots were present in the ANOVA list from Progenesis SameSpots and could be identified and excised form the gel.

Table 21. Summary of top striatum spots in rats ranked as per Mann-Whitney results in SPSS between the haloperidol and the control group treated.

Spot no.	Mann-Whitney (Progenesis) (p)	Fold change	Mann-Whitney (SPSS) (p)	SPSS rank	Label on SameSpots	ANV Control	ANV Haloperidol
877	.147	1.1	.106	2	2	4.24E+05	4.79E+05
1159	.003	1.2	.005	5	5	2.20E+06	1.81E+06
968	.253	1.1	.386	10	9	3.81E+06	3.60E+06
1113	.001	1.3	.001	13	10	1.39E+06	1.07E+06
1228	.001	1.2	.001	19	13	2.37E+06	1.98E+06
1143	.552	1	.564	23	16	4.79E+05	4.60E+05
1163	.006	1.2	.006	24	17	7.99E+05	6.65E+05
595	.309	1	.184	29	18	3.79E+06	3.63E+06
729	.182	1.1	.149	32	21	2.64E+06	2.97E+06
445	.003	1.3	.008	33	22	7.99E+05	6.29E+05
1039	.318	1.1	.326	42	28	1.62E+06	1.52E+06

These spots were present in the ANOVA list from Progenesis SameSpots and could be identified and excised from the gel. AVN = Average Normalised Volume.

Table 22. Summary of top striatum spots in rats ranked as per Mann-Whitney results in SPSS between the citalopram and the control group treated.

Spot no.	Mann-Whitney (Progenesis) (p)	Fold change	Mann-Whitney (SPSS) (p)	SPSS rank	Label on SameSpots	ANV Control	ANV Citalopram
877	.009	1.3	.007	2	2	4.24E+05	3.29E+05
1159	.001	1.4	.002	5	5	2.20E+06	1.63E+06
968	4.76E-04	1.2	.000	10	9	3.81E+06	3.11E+06
1113	.016	1.3	.014	13	10	1.39E+06	1.09E+06
1228	.005	1.2	.016	19	13	2.37E+06	2.00E+06
1143	6.05E-04	1.5	.001	23	16	4.79E+05	3.24E+05
1163	.006	1.2	.010	24	17	7.99E+05	6.44E+05
595	6.83E-04	1.2	.002	29	18	3.79E+06	3.29E+06
729	.007	1.2	.027	32	21	2.64E+06	2.23E+06
1039	.001	1.3	.005	42	28	1.62E+06	1.25E+06

These spots were present in the ANOVA list from Progenesis SameSpots and could be identified and excised from the gel. AVN = Average Normalised Volume.

Table 23. Striatum spots in rats selected for MS with average normalised volumes.

Spot No.	Mann-Whitney Progenesis (p)	Fold change	Spot label	Control	Haloperidol	Citalopram
877	3.78E-04	1.5	s1	4.24E+05	4.79E+05	3.29E+05
1159	7.92E-04	1.4	s2	2.20E+06	1.81E+06	1.63E+06
1228	.002	1.2	s3	2.37E+06	1.98E+06	2.00E+06
445	.002	1.3	s4	7.99E+05	6.29E+05	7.80E+05
729	.002	1.3	s5	2.64E+06	2.97E+06	2.23E+06
1039	.004	1.3	s6	1.62E+06	1.52E+06	1.25E+06
1163	.006	1.2	s7	7.99E+05	6.65E+05	6.44E+05
1143	.008	1.5	s8	4.79E+05	4.60E+05	3.24E+05
963	.009	1.2	s9	2.86E+06	2.54E+06	2.30E+06
1113	.009	1.3	s10	1.39E+06	1.07E+06	1.09E+06
595	.01	1.2	s11	3.79E+06	3.63E+06	3.29E+06
968	.011	1.2	s12	3.81E+06	3.60E+06	3.11E+06
1142	.013	1.2	s13	6.52E+06	5.62E+06	5.36E+06
838	.014	1.2	s14	2.45E+06	2.00E+06	2.15E+06
961	.018	1.2	s15	4.43E+06	3.91E+06	3.60E+06
1214	.021	1.2	s16	7.96E+05	6.54E+05	6.38E+05
908	.022	1.2	s17	1.51E+06	1.32E+06	1.28E+06
642	.038	1.3	s18	4.52E+06	3.56E+06	3.56E+06

The location of the spots is shown in Figure 23. These spots were excised from the gel and taken for identification to the King's College Proteomics facility. The results obtained from the LC/MS/MS procedure are summarised in Table 24.

Spots s1, s6, s9, s12, s15, and s17 were located very close to each other. Similarly s8, s2, s10 and s7 seemed to conform to another distinctive 'group'. The remaining spots, s3, s13, s14, s5, s11, s4 and s18 appeared to be more widely distributed in the gel area.

All spots were downregulated in the citalopram group with the exception of s4, which expression was similar to the control group and contained Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial (Table 25). Haloperidol upregulated the expression of s1 (aspartate aminotransferase, cytoplasmic; isocitrate dehydrogenase [NAD] subunit beta, mitochondrial; fructose-bisphosphate aldolase A; triosephosphate isomerase) and s5 (ATP synthase subunit α, mitochondrial). It did not alter the expression of s6 (voltage-dependent anion-selective channel protein 1; voltage-dependent anion-selective channel protein 2; glyceraldehyde-3-phosphate dehydrogenase; V-type proton ATPase subunit E 1) and downregulated the remaining proteins identified. Trisephosphatase isomerase was identified in s1, s2, s7 and s8. It was downregulated in all of them except in s1 in the haloperidol treated group in which it appeared to be upregulated. Glyceraldehyde-3-phosphate dehydrogenase was also downregulated in both groups and was seen in spots s6, s9, s12 and s15. Pyruvate kinase isozymes M1/M2 was present in spots s3, s11, and s12. Fructose-bisphosphate aldolase A was identified in s1 and s17 and was downregulated in all groups with the exception of the haloperidol treated one where it showed an upregulation of expression. Protein DJ-1 was detected in s3 and s12 and its expression was downregulated by both treatments. Glutathione S-transferase P was present in s3 and s16 and was downregulated in both treatment groups. Malate dehydrogenase, mitochondrial was identified in s9 and s12 and NAD-dependent deacetylase sirtuin-2 in s9 and s15.

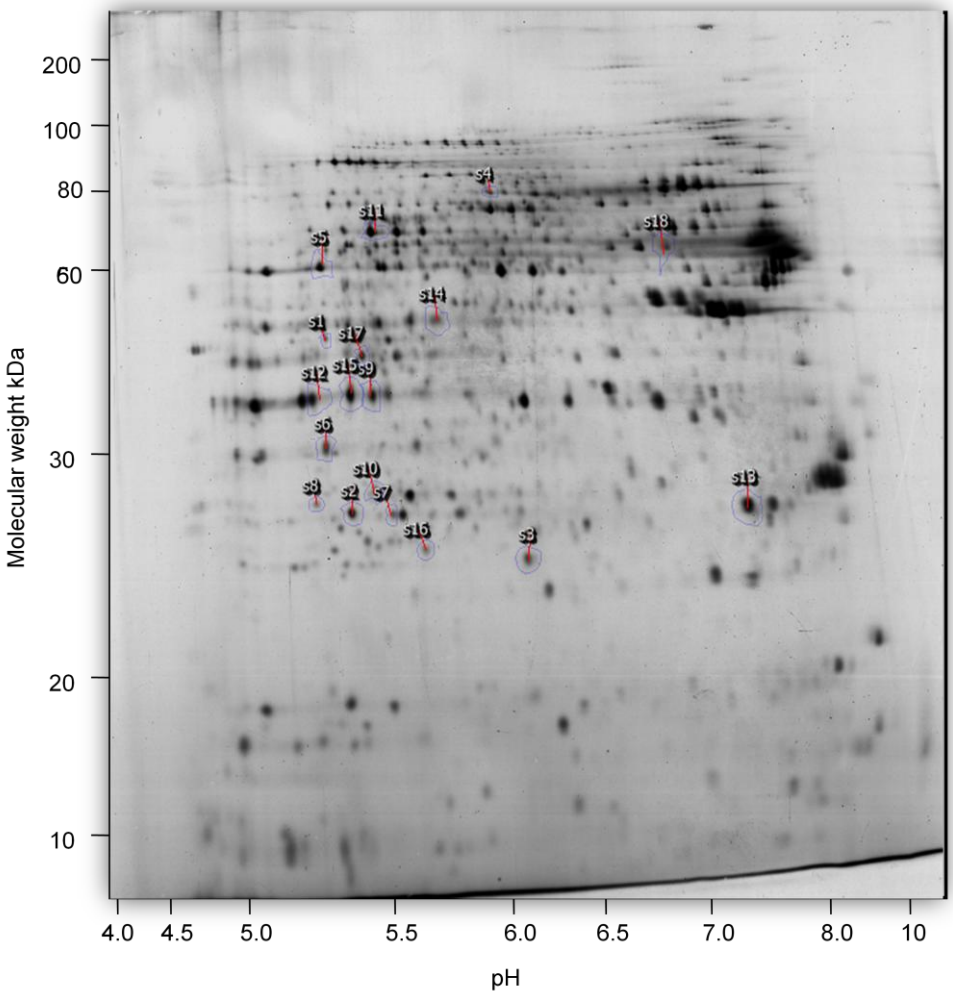

Figure 23. Selected spots in striatum for LC/MS/MS with Anova $p < .05$.

Table 24. MS results from the selected striatum spots.

Spot label (number)	Protein ID	Accession No.	MW (Da)	pI	No. Peptides Matched	No. Unique Peptides	Percentage Coverage
S1 (877)	Aspartate aminotransferase, cytoplasmic	P13221	46400	6.7	11	8	26%
	Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	Q68FX0	42327	8.9	7	4	19%
	Fructose-bisphosphate aldolase A	P05065	39327	8.3	4	3	11%
	Triosephosphate isomerase	P48500	26832	6.9	2	2	11%
S2 (1159)	Triosephosphate isomerase	P48500	26832	6.9	26	16	80%
S3 (1228)	Protein DJ-1	O88767	19961	6.3	52	19	90%
	Pyruvate kinase isozymes M1/M2	P11980	57781	6.6	13	8	22%
	Glutathione S-transferase P	P04906	23424	6.9	3	3	25%
	Acyl-protein thioesterase 1	P70470	24692	6.1	3	2	12%
S4 (445)	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	Q920L2	71570	6.8	35	21	55%
	Dihydropyrimidinase-related protein 2	P47942	62239	6.0	2	2	5%
S5 (729)	ATP synthase subunit α, mitochondrial	P15999	59717	9.2	58	32	62%
S6 (1039)	Voltage-dependent anion-selective channel protein 1	Q9Z2L0	30737	8.6	18	11	58%
	Voltage-dependent anion-selective channel protein 2	P81155	31726	7.4	14	10	48%
	Glyceraldehyde-3-phosphate dehydrogenase	P04797	35805	8.1	7	4	29%
	V-type proton ATPase subunit E 1	Q6PCU2	26112	8.4	3	3	21%
S7 (1163)	Triosephosphate isomerase	P48500	26832	6.9	36	15	77%
S8 (1143)	Dihydropteridine reductase	P11348	25536	7.7	20	12	66%
	Triosephosphate isomerase	P48500	26832	6.9	1	1	5%

Table 24 (continued)

Spot label (number)	Protein ID	Accession No.	MW (Da)	pI	No. Peptides Matched	No. Unique Peptides	Percentage Coverage
S9 (963)	Glyceraldehyde-3-phosphate dehydrogenase	P04797	35805	8.1	29	5	23%
	Malate dehydrogenase, mitochondrial	P04636	35661	8.9	6	2	8%
	NAD-dependent deacetylase sirtuin-2	Q5RJQ4	39294	6.7	3	2	10%
S10 (1113)	Carbonic anhydrase 2	P27139	29096	6.9	11	7	33%
	Phosphoglycerate mutase 1	P25113	28814	6.7	3	3	22%
S11 (595)	Pyruvate kinase isozymes M1/M2	P11980	57781	6.6	92	43	74%
S12 (968)	Glyceraldehyde-3-phosphate dehydrogenase	P04797	35805	8.1	49	11	58%
	Pyruvate kinase isozymes M1/M2	P11980	57781	6.6	8	4	13%
	Protein DJ-1	O88767	19961	6.3	6	3	26%
	Malate dehydrogenase, mitochondrial	P04636	35661	8.9	4	1	6%
S13 (1142)	Ubiquitin carboxyl-terminal hydrolase isozyme L1	Q00981	24822	5.1	39	8	55%
	Rho GDP-dissociation inhibitor 1	Q5XI73	23393	5.1	7	2	24%
	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	P19234	27361	6.2	3	2	10%
S14 (838)	Glutamine synthetase	P09606	42240	6.6	28	6	24%
	Phosphoglycerate kinase 1	P16617	44510	8.0	22	4	18%
	Pyruvate dehydrogenase E1 component subunit α, somatic form, mitochondrial	P26284	43227	8.5	4	2	5%
	Creatine kinase U-type, mitochondrial	P30275	46974	8.4	7	1	4%
S15 (961)	Glyceraldehyde-3-phosphate dehydrogenase	P04797	35805	8.1	33	7	33%
	NAD-dependent deacetylase sirtuin-2	Q5RJQ4	39294	6.7	7	5	17%

Table 24 (continued)

Spot label (number)	Protein ID	Accession No.	MW (Da)	pI	No. Peptides Matched	No. Unique Peptides	Percentage Coverage
S16 (1214)	Glutathione S-transferase P	P04906	23424	6.9	15	5	30%
S17 (908)	Fructose-bisphosphate aldolase C	P09117	39259	6.7	41	10	36%
S18 (642)	V-type proton ATPase subunit B, brain isoform	P62815	56515	5.6	61	11	34%
	Tubulin beta-2C chain	Q6P9T8	49769	4.8	19	2	7%

Table 25. Summary of direction of expression from the selected striatum spots.

Spot label (number)	Protein ID	Haloperidol	Citalopram
S1 (877)	Aspartate aminotransferase, cytoplasmic Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial Fructose-bisphosphate aldolase A Triosephosphate isomerase	↑	↓
S2 (1159)	Triosephosphate isomerase	↓	↓
S3 (1228)	Protein DJ-1 Pyruvate kinase isozymes M1/M2 Glutathione S-transferase P Acyl-protein thioesterase 1	↓	↓
S4 (445)	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial Dihydropyrimidinase-related protein 2	↓	~
S5 (729)	ATP synthase subunit alpha, mitochondrial	↑	↓
S6 (1039)	Voltage-dependent anion-selective channel protein 1 Voltage-dependent anion-selective channel protein 2 Glyceraldehyde-3-phosphate dehydrogenase V-type proton ATPase subunit E 1	~	↓
S7 (1163)	Triosephosphate isomerase	↓	↓
S8 (1143)	Dihydropteridine reductase Triosephosphate isomerase	↓	↓
S9 (963)	Glyceraldehyde-3-phosphate dehydrogenase Malate dehydrogenase, mitochondrial NAD-dependent deacetylase sirtuin-2	↓	↓
S10 (1113)	Carbonic anhydrase 2 Phosphoglycerate mutase 1	↓	↓
S11 (595)	Pyruvate kinase isozymes M1/M2	~	↓
S12 (968)	Glyceraldehyde-3-phosphate dehydrogenase Pyruvate kinase isozymes M1/M2 Protein DJ-1 Malate dehydrogenase, mitochondrial	↓	↓
S13 (1142)	Ubiquitin carboxyl-terminal hydrolase isozyme L1 Rho GDP-dissociation inhibitor 1 NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	↓	↓
S14 (838)	Glutamine synthetase Phosphoglycerate kinase 1 Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial Creatine kinase U-type, mitochondrial	↓	↓
S15 (961)	Glyceraldehyde-3-phosphate dehydrogenase NAD-dependent deacetylase sirtuin-2	↓	↓
S16 (1214)	Glutathione S-transferase P	↓	↓
S17 (908)	Fructose-bisphosphate aldolase C	↓	↓
S18 (642)	V-type proton ATPase subunit B, brain isoform Tubulin beta-2C chain	↓	↓

4.3.4. Prefrontal

The purpose of this analysis was to show that region specific changes occurred in striatum following treatment with haloperidol and citalopram. This was achieved by ensuring that there was no overlap with the striatum analysis. Prefrontal tissue images were analysed in the same way that the plasma and striatum images. Spots were taken following the same procedure; namely, the ANOVA values for all manually curated spots were calculated using the statistical package from Progenesis SameSpots v3.3 and SPSS 16.0 and both list compared for overlapping spots.

Non-parametric tests were conducted between the control and haloperidol treated groups and between control and citalopram treated groups (data not shown). Spots appearing on the four resulting lists were ranked according to statistical significance and fold change. There were 2842 identifiable spots of which 110 had an ANOVA value $p < .05$. All were manually curated and 33 were selected as being statistically significant and not an artefact of the gel. A summary of the spots selected and a table detailing the main characteristics of those is shown here (Figure 24, Table 26). The resulting spots were not sent to MS for identification. A PCA was also performed using those 33 spots, which showed some specific group distribution, but not as clearly as in the plasma or the striatum analyses (Figure 25).

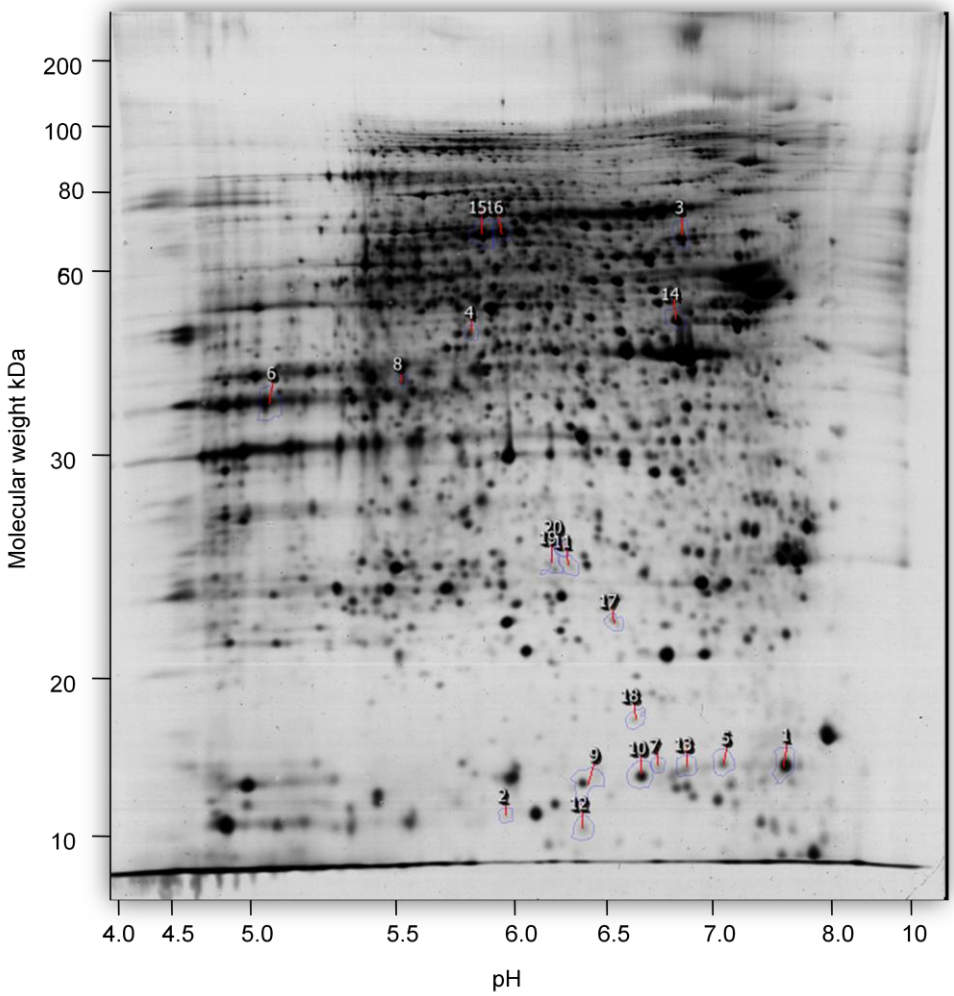

Figure 24. Selected Prefrontal spots for LC/MS/MSwith Anova p < .05.

Table 26. Prefrontal spots with average normalised volumes.

Spot No.	Anova Progenesis (p)	Fold change	Spot label	Control	Haloperidol	Citalopram
2708	.003	1.6	2	2.76E+05	4.34E+05	4.14E+05
2618	.006	1.6	5	1.23E+06	1.85E+06	2.01E+06
2621	.008	1.8	7	3.60E+05	4.37E+05	6.46E+05
1622	.01	1.3	4	1.08E+06	8.52E+05	8.63E+05
1829	.011	1.3	6	6.66E+06	5.22E+06	5.66E+06
1249	.011	1.3	3	3.29E+06	3.71E+06	2.90E+06
1587	.011	1.3	14	3.52E+06	4.53E+06	3.99E+06
2619	.011	1.3	1	4.23E+06	5.67E+06	4.88E+06
2729	.016	1.7	12	5.44E+05	7.82E+05	9.18E+05
3061	.021	1.3	15	5.20E+06	6.68E+06	5.77E+06
2650	.028	1.3	9	3.06E+06	3.86E+06	3.94E+06
755	.028	1.5	8	9.68E+05	8.76E+05	6.48E+05
3079	.029	1.3	16	3.26E+06	4.25E+06	3.55E+06
2629	.033	1.5	13	1.21E+06	1.76E+06	1.79E+06
2165	.038	1.3	20	2.89E+05	3.33E+05	3.81E+05
2348	.039	1.3	17	6.46E+05	7.69E+05	8.54E+05
2223	.042	1.3	19	6.70E+05	8.34E+05	8.73E+05
2203	.050	1.3	11	6.70E+05	7.59E+05	8.74E+05
2549	.083	1.7	18	4.07E+05	6.77E+05	6.23E+05
2638	.228	1.3	10	3.11E+06	4.00E+06	3.93E+06

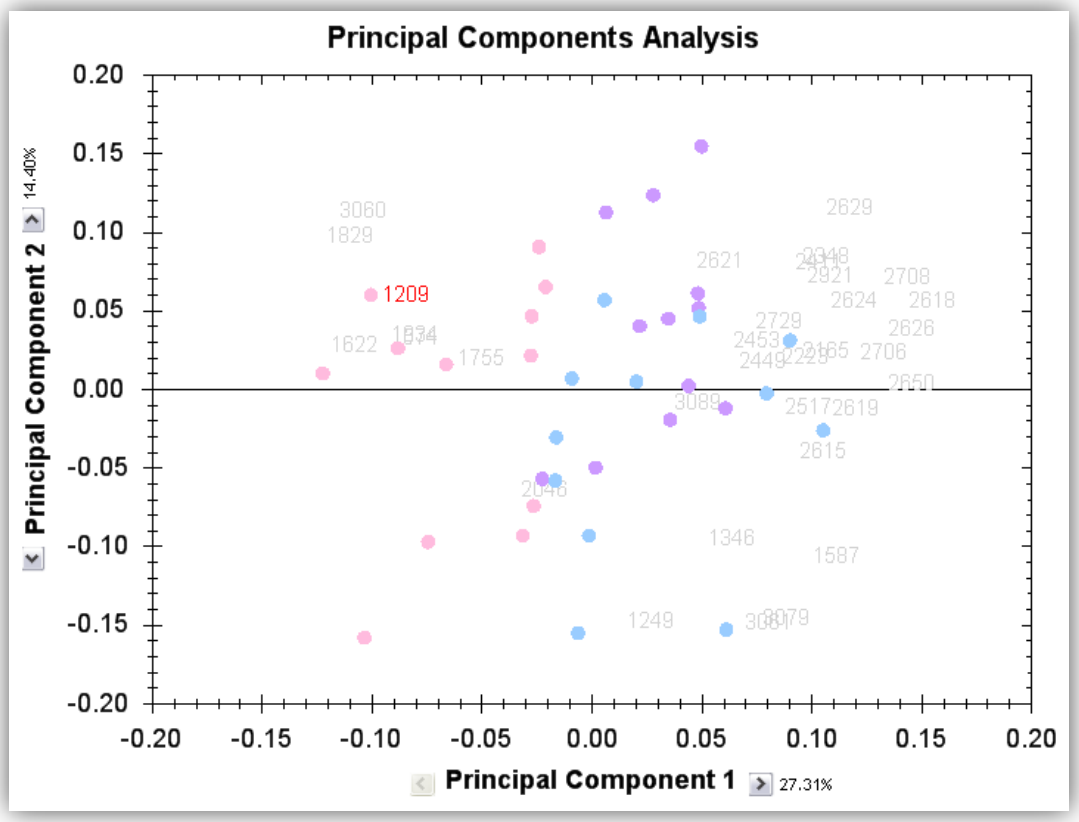

Figure 25. PCA of 33 significant spots in prefrontal included in analysis.
Principal Component 1 (27.31% of total variance). Principal Component 2 (14.40% of total variance). Control (pink), haloperidol (blue), citalopram (purple). 1209 is the spot which was given the smallest p value in the ANOVA.

4.4. Summary

This is, to the best of our knowledge, the first study conducted to explore simultaneously the effects of antipsychotic and antidepressant medication in the striatum and plasma proteome of F344 animals.

Inbred F344 animals were chosen over outbred strains like Long Evans (LE) or Wistar rats as these latter groups have greater genetic variation and variability in pathological lesions and require larger sample sizes to show significant statistical differences than inbred populations. F344 are albino animals with a Wistar background and are usually labelled as young (3-6 months), adult, (11-18 months) and aged (22-24 months). We used adult rats for our study as they maintain a moderate weight as adults (around 250 g) and are for the most part healthy and lacking the more common health problems usually present in aged F344 rats like adenomas of Leydig cells, leukaemia, pituitary adenoma, bile duct hyperplasia or hepatic micro abscesses.

We initially envisaged caging the animals in groups of three, one subject per treatment group (haloperidol, citalopram and vehicle treated) in order to minimise experimental error by ensuring that the same conditions of light, food, and water were provided homogenously throughout the cohort. Unfortunately, it was soon observed that haloperidol treated animals became oversedated, which rendered them unable to defend themselves from attacks from the other two animals in the cage, which attempted to chew on the site of the incision and pump implant. For that reason, we were forced to change the experimental conditions after the implantation of the pumps and the animals were housed according to treatment received in cages of 4 animals each. This resulted in having 3 cages of 4 animals each per treatment group and for that reason, housing conditions may have slightly differed among treatment groups, although we understand this is common and normal practice in many animal houses facilities. The sacrifice of the animals, the extraction of brain tissue and plasma as well as other tissues (lungs, kidneys, heart, liver, muscle and adipose tissue) was performed on 3 days to minimise 'batch effect'. On day 1, 4 animals were sacrificed as 'test' for the procedure first and then 4 vehicle-treated animals were processed. On day 2, the entire haloperidol-treated cohort was sacrificed alongside 4 vehicle-treated animals. On day 3, the entire citalopram-treated group and the remaining 4 vehicle treated animals were processed.

Haloperidol (4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one) is a phenyl-piperidinyl-butyrophenone used in the treatment of psychotic disorders such as SCZ and schizoaffective disorder, and is the drug of choice for Tourette syndrome. It also has sedative and antiemetic activity. Haloperidol pharmacological effects are primarily at subcortical level. It is a strong blocker of adrenergic receptors and dopaminergic receptors.

Peripherally, it exerts weaker anticholinergic activity and ganglionic blocking action. In addition, it weakly blocks histamine and serotonergic receptors.

The precise mechanism whereby the therapeutic effects of haloperidol are produced is not precisely known. It is a CNS depressor at the subcortical level of the brain and seems to act at the caudate nucleus inhibiting the ascending reticular activating system of the brain stem interrupting the impulse between the diencephalon and the cortex. Inhibition of catecholamine receptors may also contribute to haloperidol's mechanism of action.

Citalopram is a furancarbonitrile (1-[3-(dimethylamino)propyl]-1 (4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile), and an antidepressant of the serotonin uptake inhibitors' class. The therapeutic effect of citalopram is presumed to be linked to its inhibition of neuronal serotonin uptake at the serotonin reuptake pump of the presynaptic neuronal membrane, enhancing the actions of serotonin on $5HT_{1A}$ autoreceptors. citalopram has also very weak effects on norepinephrine and dopamine neuronal reuptake and no significant affinity for adrenergic (alpha1, alpha2, beta), cholinergic, GABA, dopaminergic, histaminergic, serotonergic ($5HT_{1A}$, $5HT_{1B}$, $5HT_2$), or benzodiazepine receptors. We predicted that due to these therapeutic profiles, some of the pathways that will be highlighted would be altered by the effect of both agents whereas others will be exclusively affected by either one of them.

In order to verify that the medication reached the target tissues, an ELISA assay was conducted to determine serotonin plasma and striatum levels in the entire cohort. A serotonin ELISA was used as both agents were known to alter serotonergic pathways and it was expected that striatum serotonin levels in the citalopram treated animals far exceeded those in the other groups. The fact that the haloperidol treated group showed an increase in serotonin levels with respect to controls also demonstrates that haloperidol was also effectively delivered via the implanted mini-pumps.

Brain serotonin levels were increased by citalopram and haloperidol, in the two treated groups despite early evidence of haloperidol not having an effect on the synthesis of 5-hydroxytryptophan (5-HTP), a precursor of serotonin (Carlsson *et al.* 1977). This effect could possibly be mediated by the indirect effect of haloperidol on dopa decarboxylase (Buckland *et al.* 1992) (Figure 26); whereas plasma serotonin levels were increased in the haloperidol treated group and markedly decreased in the plasma of the citalopram treated animals.

The reasons for this were not explored further in this study but remain a subject of interest and further analysis since serotonin is synthesised in serotonergic neurons throughout the nervous system (although principally in the raphe nuclei in the brain) and in enterochromaffin cells of the gastrointestinal tract. Tryptophan, an essential amino acid, is the

precursor to serotonin synthesis occurring by the sequential action of tryptophan hydroxylase to 5-hydroxytryptophan and decarboxylation into 5-HT by 5-hydroxytryptophan decarboxylase. The availability of tryptophan influences the circadian levels of serotonin in the brain and plasma (Mateos *et al.* 2009).

Serotonin is stored mainly as a peripheral pool in platelet granules and at lower levels in plasma. The principal metabolite of serotonin is 5-hydroxyindoleacetic acid generated by monoamine oxidase in hepatic and lung endothelial cells. Thus 5-HT plasma levels reflect equilibrium between synthesis, catabolism, platelet storage capacity, uptake and release kinetics and ability. It is plausible that citalopram and haloperidol have opposing effects only in peripheral serotonin levels.

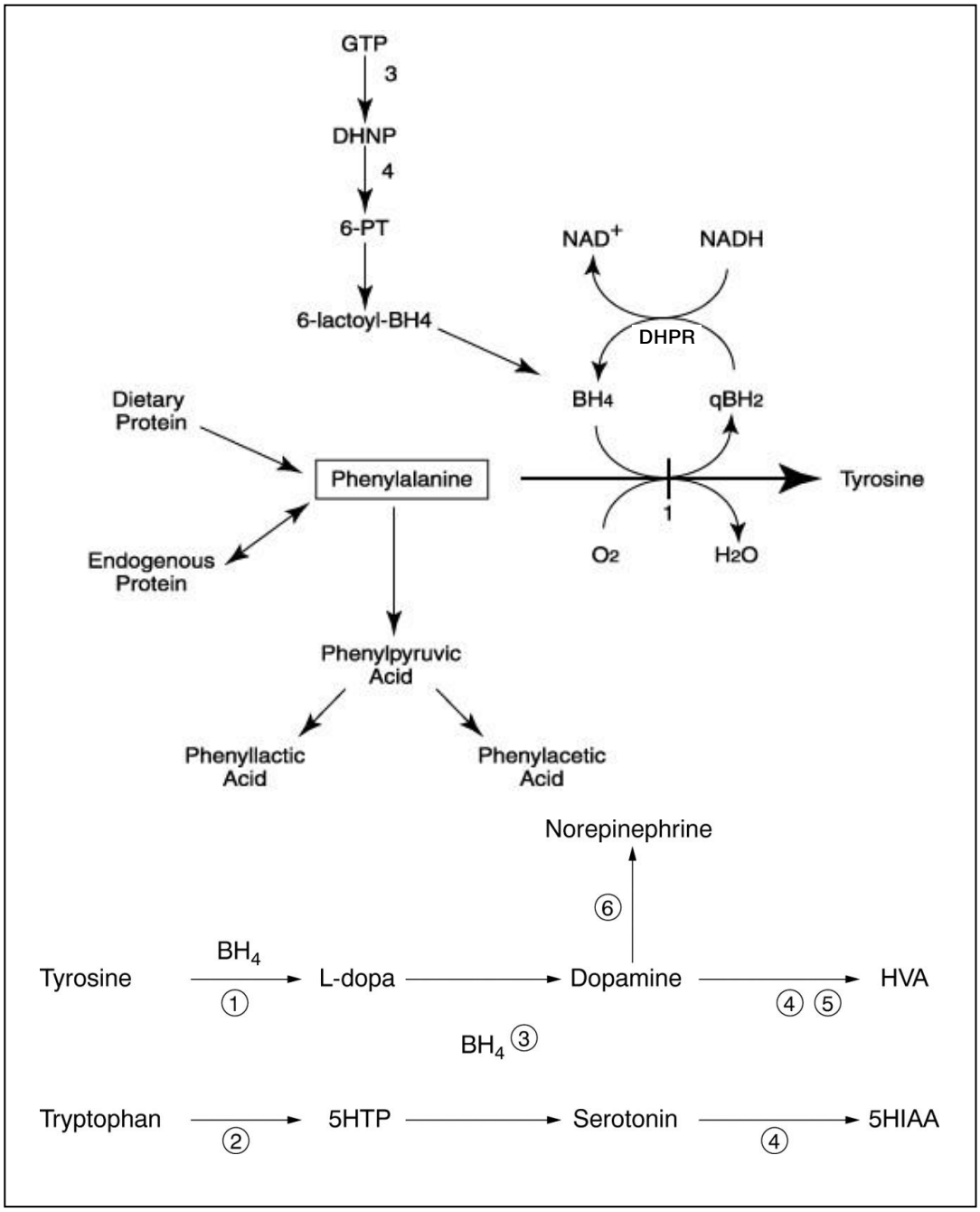

Figure 26. Metabolic pathway of dopamine, serotonin, and BH4.

5-hydroxyindole acetic acid is formed initially from tryptophan in a reaction catalysed by tryptophan hydroxylase○2 to form 5-hydroxytryptophan (5HTP), which requires molecular oxygen and tetrahydrobiopterin (BH4) for its activity. 5-hydroxytryptophan is decarboxylated by pyrimidine dependent aromatic L-amino acid decarboxylase○3 to form the active neurotransmitter serotonin. Serotonin is catabolised by monoamine oxidase○4 to form 5-hydroxyindole acetic acid (5HIAA). Tyrosine is metabolised by tyrosine hydroxylase○1 to L-dopa. Aromatic L-amino acid decarboxylase○3 is also required for the decarboxylation of L-dopa to dopamine, which is then catabolised to homovanillic acid by monoamine oxidase and catechol O-methyl-transferase○5. GTP = Guanosine triphosphate. DHNP = Dihydroneopterin triphosphate. 6-PT = 6-Pyruvoyl-tetrahydropterin. DHPR = Dihydropteridin reductase. qBH2 = Quinonoid dihydroobopterin. BH4 = tetrahydrobiopterin.

A classical proteomic approach was used to examine the two target tissues (plasma and striatum) as well as the control tissue (prefrontal). Twenty two different proteins were identified in 19 plasma spots (Table 27): α-1-antiproteinase; α-1-macroglobulin; angiotensinogen; anionic trypsin-2; apolipoprotein A-IV; ATP synthase subunit beta, mitochondrial; beta-2-glycoprotein 1; carbonic anhydrase 1; carbonic anhydrase 2; complement C3; fibrinogen α chain; fibrinogen beta chain; fibrinogen gamma chain; haptoglobin; Ig gamma-2B chain C region; Ig kappa chain C region, A allele; Ig lambda-2 chain C region; plasminogen; pyruvate kinase isozymes M1/M2; serotransferrin; tubulin α-1C chain and vitamin D-binding protein.

And 30 were identified in 18 striatum spots (Table 27): acyl-protein thioesterase 1; aspartate aminotransferase, cytoplasmic ; ATP synthase subunit α, mitochondrial; carbonic anhydrase 2; creatine kinase U-type, mitochondrial; dihydropteridine reductase; dihydropyrimidinase-related protein 2; fructose-bisphosphate aldolase A; fructose-bisphosphate aldolase C; glutamine synthetase; glutathione S-transferase P; Glyceraldehyde-3-phosphate dehydrogenase; isocitrate dehydrogenase [NAD] subunit beta, mitochondrial ; malate dehydrogenase, mitochondrial; NAD-dependent deacetylase sirtuin-2; NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial; phosphoglycerate kinase 1; phosphoglycerate mutase 1; protein DJ-1; pyruvate dehydrogenase E1 component subunit α, somatic form, mitochondrial; pyruvate kinase isozymes M1/M2; Rho GDP-dissociation inhibitor 1; succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial; triosephosphate isomerase; tubulin beta-2C chain; Ubiquitin carboxyl-terminal hydrolase isozyme L1; voltage-dependent anion-selective channel protein 1; voltage-dependent anion-selective channel protein 2; V-type proton ATPase subunit B, brain isoforms and V-type proton ATPase subunit E1.

Table 27. Proteins identified in plasma and striatum of F344 rats.

Proteins in plasma	Accession No.	Proteins in striatum	Accession No.
Alpha-1-antiproteinase	p17475	Acyl-protein thioesterase 1	p70470
Alpha-1-macroglobulin	q63041	Aspartate aminotransferase, cytoplasmic	p13221
Angiotensinogen	p01015	ATP synthase subunit α, mitochondrial	p15999
Anionic trypsin-2	p00763	Carbonic anhydrase 2	p27139
Apolipoprotein A-IV	P02651	Creatine kinase U-type, mitochondrial	p30275
ATP synthase subunit beta, mitochondrial	p10719	Dihydropteridine reductase	p11348
Beta-2-glycoprotein 1	p26644	Dihydropyrimidinase-related protein 2	p47942
Carbonic anhydrase 1	b0BNN3	Fructose-bisphosphate aldolase A	p05065
Carbonic anhydrase 2	p27139	Fructose-bisphosphate aldolase C	p09117
Complement C3	p01026	Glutamine synthetase	p09606
Fibrinogen alpha chain	p06399	Glutathione S-transferase P	p04906
Fibrinogen beta chain	p14480	Glyceraldehyde-3-phosphate dehydrogenase	p04797
Fibrinogen gamma chain	p02680	Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	q68FX0
Haptoglobin	p06866	Malate dehydrogenase, mitochondrial	p04636
Ig gamma-2B chain C region	p20761	NAD-dependent deacetylase sirtuin-2	q5RJQ4
Ig kappa chain C region, A allele	p01836	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	p19234
Ig lambda-2 chain C region	p20767	Phosphoglycerate kinase 1	p16617
Plasminogen	q01177	Phosphoglycerate mutase 1	p25113
Pyruvate kinase isozymes M1/M2	p11980	Protein DJ-1	o88767
Serotransferrin	p12346	Pyruvate dehydrogenase E1 component subunit α, somatic form, mitochondrial	p26284
Tubulin alpha-1C chain	q6AYZ1	Pyruvate kinase isozymes M1/M2	p11980
Vitamin D-binding protein	p04276	Rho GDP-dissociation inhibitor 1	q5XI73
		Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	q920L2
		Triosephosphate isomerase	p48500
		Tubulin beta-2C chain	q6P9T8
		Ubiquitin carboxyl-terminal hydrolase isozyme L1	q00981
		Voltage-dependent anion-selective channel protein 1	q9Z2L0
		Voltage-dependent anion-selective channel protein 2	p81155
		V-type proton ATPase subunit B, brain isoform	p62815
		V-type proton ATPase subunit E1	q6PCU2

Antagonistic effects between haloperidol and citalopram were observed in both plasma and striatum.

In plasma, haloperidol decreased the expression of fibrinogen alpha, beta and gamma chains; beta-2-glycoprotein 1; Ig gamma-2B chain C region; serotransferrin; anionic trypsin-2; pyruvate kinase isozymes M1/M2; complement C3; apolipoprotein A-IV and ATP synthase subunit beta, mitochondrial; while citalopram increased it. The expression of α-1-mocroglobulin was increased by haloperidol and decreased by citalopram in plasma (Table 28).

In striatum, haloperidol increased the expression of aspartate aminotransferase, cytoplasmic; isocitrate dehydrogenase [NAD] subunit beta, mitochondrial; fructose-bisphosphate aldolase A; triosephosphate isomerise and ATP synthase subunit α, mitochondrial; while citalopram decreased it (Table 29).

The main pathways in which haloperidol and citalopram showed opposing expression effects are glucose and lipid metabolism, mitochondrial function, immune response, coagulation, calcium signalling and apoptosis.

Table 28. Plasma proteins for which haloperidol and citalopram showed oppossed expression profile.

Protein I.D.	Accession No.	Gene Symbol	Function
Alpha-1-macroglobulin	q63041	Pzp	Belongs to the protease inhibitor I39 (α-2-macroglobulin) family.
Anionic trypsin-2	p00763	Prss2	Calcium ion binding, peptidase activity.
Apolipoprotein A-IV	P02651	Apoa4	Antioxidant, cholesterol transporte and lipid binding
ATP synthase subunit beta, mitochondrial	p10719	Atp5b	MHC class 1 protein , calcium ion and ATP binding
Beta-2-glycoprotein 1	p26644	Apoh	Glycoprotein and lipid binding. May prevent activation of intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells
Complement C3	p01026	C3	Lipid and protein binding activity. Activation of the complement system
Fibrinogen alpha chain	p06399	Fga	Blood coagulation, platelet activation, response to calcium ion
Fibrinogen beta chain	p14480	Fgb	Platelet activation, response to calcium ion
Fibrinogen gamma chain	p02680	Fgg	Inflammatory and calcium ion response, platelet activation
Haptoglobin	p06866	Hp	Inflammatory response, haemoglobin binding, apoptosis
Ig gamma-2B chain C region	P20761	Igh	Antigen binding
Pyruvate kinase isozymes M1/M2	p11980	Pkm2	Glucose metabolic processes, apoptosis, response to hypoxia
Serotransferrin	p12346	Tf	Iron ion transport, regulation of myelination and cell proliferation

Table 29. Striatum proteins for which haloperidol and citalopram showed oppossed expression profile.

Proteins	Accession No.	Gene Symbol	Function
Aspartate aminotransferase, cytoplasmic	p13221	Got1	Aminoacid metabolism
ATP synthase subunit alpha, mitochondrial	p15999	Atp5a1	MHC class 1 protein, ATP and hydrogen ion binding
Fructose-bisphosphate aldolase A	p05065	Aldoa	Glycolisis and gluconeogenesis, scaffolding protein
Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	q68FX0	Idh3B	Oxidorreductase activity, metabolism of brown adipose tissue
Triosephosphate isomerase	p48500	Tpi1	Glycolisis and gluconeogenesis

CHAPTER 5: Finding biomarkers for psychosis in plasma

5.1. Rationale and aims

Proteome-based plasma biomarkers for neuropsychiatric conditions have been identified in disorders such as Alzheimer's (Hye *et al.* 2006) and Parkinson's disease (Chen *et al.* 2011) using classical proteomic approaches such as 2DPAGE and immunoblotting. The aim of this pilot study was to test the viability of using proteomic techniques, namely 2DPAGE coupled with LC/MS/MS, to search for biomarkers in plasma in a small, yet well characterised and matched case-control sample of patients with schizophrenia.

5.2. Material and methods

The Genetics and Psychosis (GAP) study is an infrastructure project supported by the Maudsley Charitable Fund and the National Institute of Health Research (NIHR) Biomedical Research Centre (BRC) for Mental Health. It was approved by the local ethics committee and aims to recruit all patients with a first onset psychosis attending the South London and Maudsley Mental Health National Health Service (NHS) Foundation Trust, the largest mental health trust in the UK.

Patients aged 18–65 years with a clinical diagnosis of psychosis who presented for the first time to the Lambeth, Southwark and Croydon adult in-patient units of the South London and Maudsley Mental Health National Health Service (NHS) Foundation Trust between 2005 and 2008 were approached for inclusion in the study. Written consent to provide biological samples and to allow the use of those for proteomic studies was obtained in all of those who agreed to take part.

5.3. Subjects and samples

Cases with a diagnosis of organic psychosis, learning disability, head injury, or with poor command of English language were excluded. Those who met ICD–10 criteria for a diagnosis of psychosis (codes F20–F29 and F30–F33) (World Health Organization. The ICD–10 Classification of Mental and Behavioural Disorders. Clinical Description and Diagnostic Guidelines. WHO, 1992) were invited to participate in the study. Controls were recruited over the same period of time from the local communities from where the cases lived were recruited by means of advertisements in the local press, and leaflet distribution at train stations, shops and job centres. The control sample was selected to match as much as possible the patient sample in age, gender, ethnicity, educational qualifications and employment status. Potential control subjects were excluded if they met criteria for psychosis, either by means of reporting past psychiatric history of a psychotic disorder or by being identified as possibly having one by the

Psychosis Screening Questionnaire (Bebbington PE, Nayani T. The Psychosis Screening Questionnaire. Int J Methods Psychiatr Res 1995; 5: 11 –9).

Systematic assessments on all patients were made by trained researchers to collect clinical variables using standardised research scales. All medication and non-prescription drugs used were carefully noted. Subjects were drawn from the GAP sample on the basis of having a control matched as close as possible for ethnicity, age, gender, current use of alcohol, tobacco and cannabis. Potential cases were further selected according to the antipsychotic treatment they were receiving at the time their blood sample was obtained.

For this study, 30 cases and 30 matched controls were selected for the discovery phase. There were no statistically significant differences between the groups in terms of gender, age, ethnicity, alcohol, and tobacco and cannabis use (Table 30). A more detiled description of the sample is provided in Table 31.

Blood samples were collected using a 23G butterfly needle and a vacutainer needle holder following standard venipuncture in the cubital region, in the area of anastomosis between the radial and the ulnar veins, or in the brachial vein proximal to this area. To avoid coagulation of the sample, a 9ml K3EDTA tube (Greiner Bio-One, Cat # 455036X - EDTA tube, lavender lid) was used. Samples were kept at +4°C until being processed, with the time between collection and processing of the sample never exceeding two hours. Tubes were labelled with the subject barcode only. Blood samples were centrifuged at 3000 rcf (xg), 8 minutes at +4°C and plasma was aliquoted in 0.5 ml eppendorf tubes and immediately frozen at -80°C.

Table 30. Summary of demographic details for the subjects used for the pilot study.

		Cases Group (N = 30)		Control Group (N = 30)				
		Mean	**SD**	**Mean**	**SD**	**U**	**z**	**p**
Age		32.83	9.128	33.43	9.673	440.000	-.148	.882
		N	**%**	**N**	**%**	**χ^2**	**df**	**p**
Female		6	20	7	23.3	.098	1	.754
Ethnicity	White	16	53.3	19	63.3	.868	3	.833
	Black African	5	16.6	3	10			
	Black Caribbean	5	16.6	4	13.3			
	Other	4	13.3	4	13.3			
Alcohol Use		20	66.6	24	80	1.364	.243	.382
Tobacco Use		16	53.3	15	50	.067	1	.796
Cannabis Use		19	63.3	19	63.3	.000	1	1

M = Male, F = Female, C = Caucasian, B = Black.

Table 31. Subjects from the GAP sample used for the pilot study.

Case Control	Gender	DoB	Cannabis	Smoker	Alcohol	Ethnicity	Case medication
LGAP137	F	10.11.83	No	No	Yes	BA	Med-free
SGAP268	M	29.07.83	Yes	Yes	Yes	BA	
SGAP208	M	13.09.76	No	Yes	Yes	BA	Med free
SGAP236	M	15.04.81	Yes	Yes	Yes	BA	
OGAP137	F	13.11.81	No	No	Yes	WB	Med free
SGAP308	M	26.04.78	Yes	Yes	Yes	OC	
SGAP285	M	19.06.68	No	No	Yes	WB	Med free
SGAP168	M	09.09.64	Yes	Yes	Yes	OC	
SGAP218	M	24.05.78	Yes	Yes	No	OC	Med free
SGAP337	M	21.07.72	No	No	Yes	OC	
CGAP119	F	16.02.58	No	No	No	BC	Med free
SGAP279	F	28.11.64	Yes	Yes	Yes	BC	
CGAP122	M	08.10.77	Yes	Yes	Yes	BC	Med free
SGAP316	F	20.11.84	No	No	Yes	BC	
SGAP116	M	08.01.70	Yes	Yes	No	BA	Med free
SGAP315	M	13.11.63	Yes	Yes	Yes	Mixed	
SGAP195	M	13.10.84	Yes	Yes	Yes	OC	Med free
SGAP349	M	24.05.83	Yes	Yes	Yes	WB	
OGAP101	M	22.12.70	Yes	Yes	Yes	Mixed	Med free
SGAP346	M	23.10.76	Yes	Yes	Yes	OC	
SGAP193	M	30.06.75	Yes	Yes	Yes	OC	Olanzapine
SGAP262	M	13.01.76	Yes	Yes	Yes	OC	
CGAP130	M	04.12.86	Yes	Yes	Yes	WB	Olanzapine
SGAP311	M	06.11.86	Yes	No	No	WB	
SGAP117	M	05.10.78	Yes	No	No	WB	Olanzapine
SGAP163	F	26.04.75	Yes	Yes	Yes	WB	
SGAP136	M	19.06.63	No	No	Yes	BC	Olanzapine
SGAP260	M	07.04.59	No	No	Yes	BC	
SGAP204	M	11.12.81	Yes	No	Yes	OC	Olanzapine
SGAP183	M	14.08.81	Yes	No	Yes	OC	
SGAP232	F	02.12.75	Yes	No	No	WB	Olanzapine
SGAP253	M	13.12.65	Yes	No	Yes	WB	
EGAP113	M	28.08.68	Yes	No	No	WB	Olanzapine
SGAP131	M	13.12.65	Yes	No	Yes	WB	
SGAP110	M	12.03.53	No	No	No	WB	Olanzapine
SGAP197	M	08.01.58	No	No	Yes	OC	
OGAP135	M	22.05.79	Yes	Yes	Yes	BA	Olanzapine
EGAP103	M	19.09.80	No	No	Yes	Black Other	
CGAP132	M	21.03.77	Yes	Yes	Yes	WB	Olanzapine
SGAP283	M	01.04.75	No	No	No	WB	
SGAP169	F	18.05.69	No	No	Yes	WB	Risperidone
SGAP277	F	30.05.71	No	No	No	OC	
LGAP134	M	19.05.83	Yes	Yes	Yes	Mixed	Risperidone
SGAP280	M	30.08.81	No	No	Yes	Mixed	
SGAP154	M	04.07.74	Yes	Yes	No	Indian	Risperidone
SGAP331	M	15.03.74	No	No	No	Other Asian	
SGAP233	F	19.07.53	No	Yes	No	BC	Risperidone
SGAP102	F	17.01.52	No	Yes	No	BC	
LGAP136	M	16.05.85	Yes	No	Yes	Mixed	Risperidone
SGAP306	F	22.08.86	No	No	No	Mixed	
SGAP157	M	06.06.81	No	Yes	Yes	WB	Risperidone
SGAP338	M	03.03.81	Yes	Yes	Yes	WB	
SGAP199	M	17.03.87	Yes	Yes	Yes	WB	Risperidone
SGAP343	M	31.05.87	Yes	Yes	Yes	WB	
SGAP111	M	02.12.64	No	No	No	WB	Risperidone
SGAP345	M	16.11.58	Yes	Yes	Yes	WB	
SGAP319	M	22.12.73	Yes	No	Yes	BA	Risperidone
SGAP342	M	28.11.71	Yes	No	Yes	WB	
SGAP175	M	17.08.76	Yes	Yes	Yes	BC	Risperidone
SGAP205	F	30.06.78	Yes	Yes	Yes	OC	

BA = Black African, WB = White British, BC = Black Caribbean; OC = Other Caucasian.

5.4. Protein analysis

Since protein concentration is chiefly due to albumin content and differences in its concentration had not been reported in SCZ, loading the same protein quantity, rather than the same volume was favoured. Protein concentrations of plasma samples were determined according to the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard. In brief, samples (2 μl) were mixed with Bradford reagent (1 ml) and the optical density (OD) was read after 20 minutes at 595 nm.

Samples were defrosted on ice to minimise protein degradation and then mixed (vortex) and centifuged to homogenize the contents. The volume of plasma determined to contain 50 μg of protein (approx. 1 μl) was dissolved in IPG rehydration buffer containing 15 μl IPG Buffer (pH3-11 NL). For running 18cm Immobiline DryStrips, 350 μl total volume was required per strip. The sample comprised no more than 100 μl of the total volume and hence, eppendorfs containing 350 μl – $X_{\text{sample volume}}$ of IPG rehydration buffer + $X_{\text{sample volume}}$ were prepared and left at room temperature for 30 min to allow protein denaturation, followed by a 5 min spin at 13000 rpm.

Immobiline pH gradient strips of 18 cm length (pH 3-11) were then focused to immobilise individual proteins according to their isoelectric point, completing the first step of the process of separating the complex biological mixture. The strips were rehydrated and focused using the IPGphor™ (Amersham Bioscience) platform.

The strips were taken off the strips holders and frozen overnight at -80°C prior to the second dimension separation. Immobiline strips were taken off the freezer and thawed at RT before being equilibrated in order to re-solubilise the proteins and to reduce -S-S- bonds. Equilibration took place in an orbital shaker where they were individually placed in cylindrical glass tubes. SDS-PAGE 2D Acrylamide gels were made in-house using the Ettan Dalt II gel caster system (Amersham biotech, UK) and electrophoresis plates. A 10% acrylamide solution was made immediately before use and poured into the caster. The gels were allowed to polymerise overnight.

The IPG gel strips were cut to size at both ends, taking care not to alter the hydrated gel and inserted between the two glass sheets of each plate and gently slid down until reaching the upper surface of the polymerise gel where they were sealed in place using a solution containing agarose. Electrophoresis was performed using the Ettan Dalt™ II (Amersham Biosciences) separation unit. The gels were kept at a constant current of 5 watts per gel for 1h and then subjected to a current of 80 watts per gel for up to 10-12 h or until the

bromophenol dye used in the sealing solution had completed its run across the vertical axis of the gel. The electrophoresis separation unit was kept at RT.

Gels were stained using the silver staining procedure with an optimised and adapted version of the protocol described by Hochstrasser *et al*. (Hochstrasser *et al.* 1988). For preparative mass spectrometry silver staining was performed using a separate staining kit with slight modifications (Silver Plus one, Amersham Biosciences). Gels were scanned immediately after being developed using a Bio-Rad GS-710 Calibrated Imaging Densitometer and proprietary software (Bio-Rad, Seoul). Images of individual gels were cropped and saved in .tiff format for posterior image analysis which was performed using Progenesis Samespots software (Nonlinear Dynamics, UK) version 3.0.

Statistical analysis was performed using SPSS (v15). The mean integrated optical densities of spots on 2D gels were analysed using Student's t-test or non-parametric tests (Mann-Whitney) if not normally distributed. False Discovery Rate (FDR) correction for multiple testing was applied. Spots were selected according to significance level (p) and fold change. Validation by immunoblotting was performed on an extended sample of cases and controls. Samples were run in triplicate and the optical densities were normalized using the average density of the membrane. Average values for the repeated measures of the same sample were obtained and Student's t-test was applied in normally distributed variables, otherwise the Mann-Whitney test was used.

Selected spots were excised from 2-DGE preparatory gels containing 300 µg of protein gels and stained with modified Plus One silver stain kit (Amersham, UK) to make silver staining compatible with MS procedures. A reference image with the selected spots was printed using Progenesis SameSpots software and used as template for manual picking. The gel fragments were subsequently taken to Proteome Science plc and processed by Steve Lynham. Gel fragments were subjected to in-gel reduction, alkylation and digestion with trypsin. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the m/z and the charge state of the peptide. The mass spectral data was processed into peak lists and searched against the Swiss Prot or NCBI non-redundant databases using Mascot software (Matrix Science, UK). The data was searched using specific amino acid modification parameters, ie. Variable cysteine carbamidomethylation modification (resulting from reduction and alkylation reaction) and variable methionine oxidation modification. Sequence information was obtained for all the peptides included in the results. Protein assignments were based on the matching of MS/MS spectra to the protein sequence. All results were visually verified to ensure that the fragment ions correctly matched the assigned sequence.

The sequence coverage for each identified protein is given in Appendix 3. Four proteins were identified with two peptides matched to the protein sequence and for a further four proteins, identification was based on a single matched peptide. In these cases, the observed MW (molecular weight) and pIs (Isoelectric points) of the protein spot can be taken into account to give extra confidence to the protein sequence. From assessing the theoretical MW and pIs of the proteins to the observed values, all proteins appeared to fall within acceptable ranges. To add extra confidence to the observed results, the peptide sequence for each protein identification was subjected to an NCBI protein-protein BLAST search to ascertain that such sequences were unique to that protein. In all cases the peptides were unique to the protein indicated.

To add even further confidence to the assignments and give an indication of the false-positive rate within the dataset, each dataset was searched against a database containing the Swiss-Prot database in reverse sequence. No false-positives were assigned within the datasets indicating a high confidence in the genuine assignments.

5.5. Immunoblotting and validation of targets

Western blot was used to detect proteins of interest in an independent sample of subjects. Western blot is used to separate proteins according to molecular weight by means of gel electrophoresis. The separated proteins are then transferred to a membrane where they are incubated with specific antibodies. The validation sample was drawn from the GAP study.

Plasma proteins were resolved in 10% (w/v) polyacrylamide SDS-PAGE using the Gel unit vertical complete for precast or handcast gels up to 200mm x 100mm (Fisherbrand, FB69602). Sample loading volume was 10 μl and gels were run at constant voltage (200V, 3.00 A and 300 W) with a Bio-Rad PowerPac 300 until the dye reached the bottom end of the gel (1-2 hours). Once protein separation occurred in the acrylamide gels, the proteins were transferred to 17x6 cm pieces of 0.45 μm nitrocellulose membrane (Schleicher & Scheull) in order to be probed with specific antibodies. A Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) was used. A current of constant voltage of 100 V was then applied for 1 hour. Following the transfer, the membrane was incubated with Ponceau solution for 1 min to determine the efficiency of the protein transfer and subsequently blocked to reduce non-specific binding by using a 5% non-fat milk 0.01 M PBS solution.

Membranes were then incubated overnight at 4°C in blocking buffer with the primary antibody at an optimized dilution for detection based on manufacturer's protocol. The following morning, the membranes were washed in 0.1% PBS-Tween (3x5 min) and place in a

white, non-transparent box containing 10 ml of 0.1% PBS Tween + 5% milk for 30 min at room temperature. Secondary antibodies were diluted in 5% Milk PBSTween solution (1:6000) in a Falcon tube protected from light with foil paper. The 0.1% PBSTween+5% milk of the boxes containing the membranes was replaced with the secondary AB solution and the boxes were placed in an orbital rocker for 1 hour at RT. The membranes were washed on PBSTween 3x 5 minutes and scanned using a LI-Cor Oddissey scanner and proprietary software was used for fluorescence detection.

5.6. Results

Image analysis of the gels using Progenesis SameSpots yielded 1481 differentially expressed spots. Out of those 77 were significantly different according to p value and fold change. Principal Component Analysis (PCA) is a multivariate analysis related to factor analysis which was used to transform the observations (spots identified) in a set of uncorrelated variables (Principal Components). The first component account for as much of the variability as possible. Subasequent ones have the highest possible variance as long as they remain uncorrelated with the preceding component. Following PCA, a group distribution was observed in the sample (Figure 27) showing that cases and controls could be discriminated on the basis of the selected significantly differentially expressed spots. Eight spots (60, 61, 67, 72, 92, 94, 1286 and 1847) were selected for LC/MS/MS according to their level of significance and fold change (Figure 28). The results of the LC/MS/MS analysis and database searches are given in Table 32.

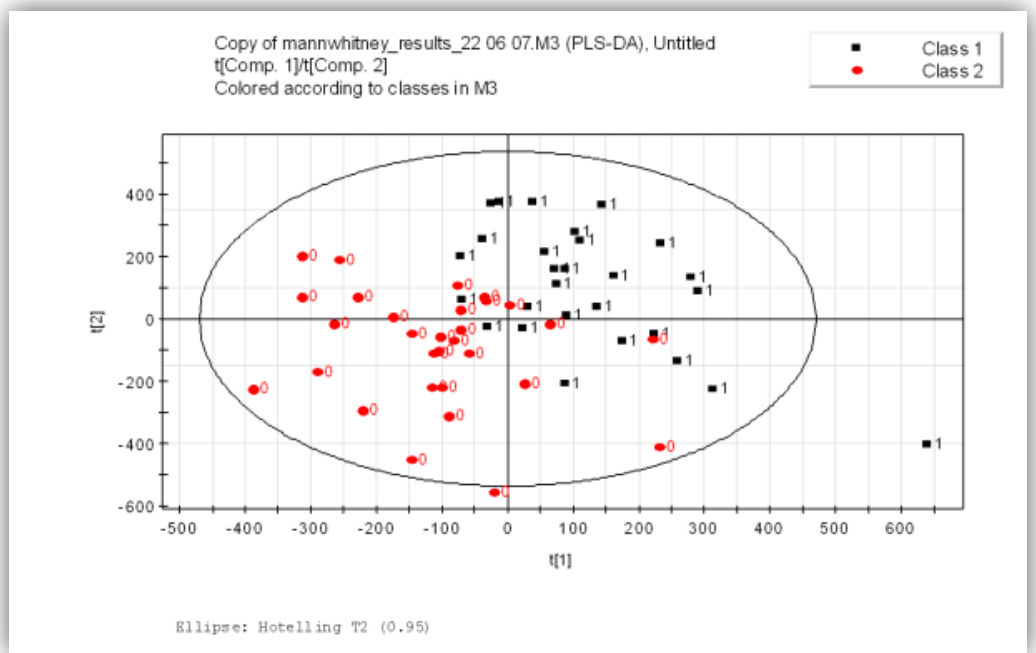

Figure 27. PCA of controls (Class 1) and cases (Class 2) showing a clear group distribution.

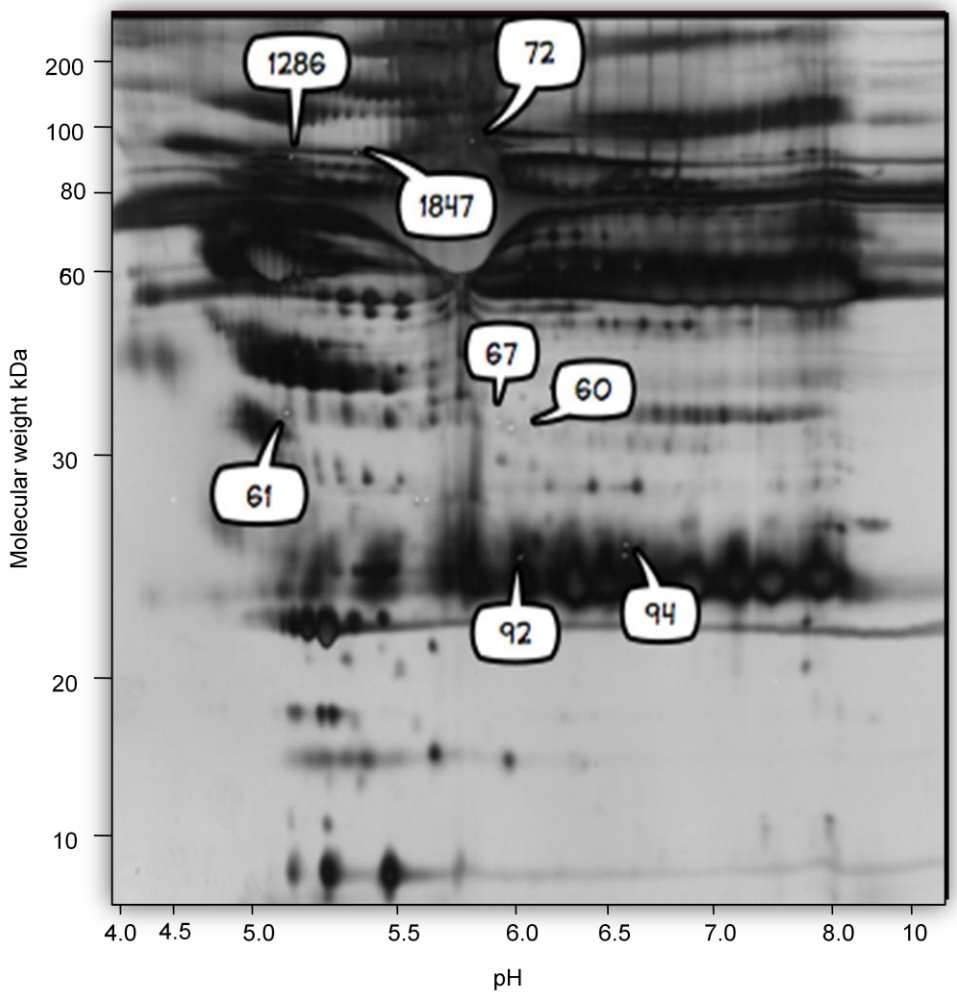

Figure 28. Image of a preparatory human plasma gel with the selected spots highlighted and excised for LC/MS/MS.

A preparatory gel, rather than an 'analytical gel' has been used here for illustration purposes. The gel is clearly overloaded and one can see the places where the gel has been 'punched out' for analysis.

Table 32. Results of LC/MS/MS analysis selected 2D gel spots in human plasma.

Spot No.	Mean Cases	Mean Controls	p-value	Fold change	Protein I.D.	Accession No.	MW (Da)	pI
60	928.01	670.06	.02	1.4	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin)	P68871	15988	6.8
					Peroxiredoxin-1 (Thioredoxin peroxidase 2)	Q06830	22096	8.3
61	2415.93	1824.78	.02	1.4	Complement factor I precursor (C3B/C4B inactivator)	P05156	65677	7.7
					Clusterin precursor (Complement-associated protein SP-40,40)	P10909	52461	5.9
67	824.49	584.70	.03	1.4	Protein S100-A8 (S100 calcium-binding protein A8) (Calgranulin-A)	P05109	10828	6.5
72	1343.12	2053.97	.05	1.3	Serum albumin precursor	P02768	69321	5.9
					Gelsolin precursor (Actin-depolymerizing factor) (ADF) (Brevin) (AGEL)	P06396	85644	5.9
92	10960.74	12721.52	.03	1.1	Ig kappa chain C region	P01834	11602	5.6
					Ig kappa chain V-II region TEW	P01617	12308	5.7
94	9663.57	8713.63	.01	1.1	Ig kappa chain C region	P01834	11602	5.6
					Ig lambda chain C regions	P01842	11230	6.9
1286	1484.57	1236.78	.05	1.3	Afamin precursor (Alpha-albumin) (Alpha-Alb)	P43652	69024	5.6
1847	4048.71	3547.80	.04	1.1	Complement C1r subcomponent precursor	P00736	80122	5.9
					Complement C4-A precursor (Acidic complement C4)	P0C0L4	192650	6.7

Thus, six spots containing hemoglobin subunit beta; peroxiredoxin-1; complement factor I precursor; clusterin precursor; S100-A8; Ig kappa chain C region; Ig lambda chain C regions; afamin precursor; complement C1r subcomponent precursor and complement C4-A precursor appeared to be increased in cases whereas the expression of serum albumin precursor; gelsolin precursor; Ig kappa chain C region and and Ig kappa chain V-II region TEW was decreased.

Commercial antibodies (Table 33) were obtained for afamin, S100A8, gelsolin, clusterin and peroxiredoxin-1 and validation was performed for these proteins.

Antibodies were tested and optimized according to manufacturer's instructions. S100A8 and gelsolin showed clear distinction bands at the correct molecular weight and therefore were selected for further analysis (Figure 29).

Immunoblots for gelsolin were performed in triplicate. Fluorencence signal intensities were individually measured (K counts) and normalised. An independent Samples t-test for equality of means was applied using SPSS v15.

Gelsolin levels were quantified in triplicate for 87 controls and 125 cases. The S100A8 validation was done in a sample of 58 controls and 82 cases. The S100B ELISA in human plasma samples was done in a sample of 75 cases and 75 matched controls.

Individual measures were normalized and the mean of the three quantifications was obtained. Student's t-test was used as the variable appeared to be normally distributed. A significant difference between cases and controls was observed. The figures presented here are the average normalised volumes for each subject. Equal variances were not assumed following a non-significant result on the Levene's Test (0.292) and the p value was calculated at $p = .01$ (Table 34 and Figure 30).

Table 33. Antibodies used for Western blot in human plasma samples.

Protein I.D.	Antibody used	Immunogen sequence	Dilution used	Host
S100A8	S100A8 purified MaxPab (B01P) (Abnova)	MLTELEKALNSIIDVYHKYSLIKGNFHAVYRD DLKKLLETECPQYIRKKG ADVWFKELDINTDGAVNFQEFLILVIKMGV AAHKKSHEESHKE	1:1000	Mouse polyclonal
Afamin	AFM (ab49139) (Abcam)	GQCIINSNKD DRPKDLSLRE GKFTDSENVC QERDADPDTF FAKFTFEYSR	1:1000	Rabbit polyclonal
Peroxiredoxin 1	Peroxiredoxin 1 (ab15571) (Abcam)	Synthetic peptide: LVSDPKRTIAQD	1:1000	Rabbit polyclonal
Clusterin	Anti-Apolipoprotein J (ab16077)	Full length native protein (purified) (Human)	1:1000	Mouse monoclonal
Gelsolin	Gelsolin (ab11081) (Abcam)	The antibody is directed against an epitope located on the 47 kD peptide derived from a chymotryptic cleavage of gelsolin. This peptide contains the carboxy terminal actin binding site for gelsolin.	1:1000	Mouse monoclonal

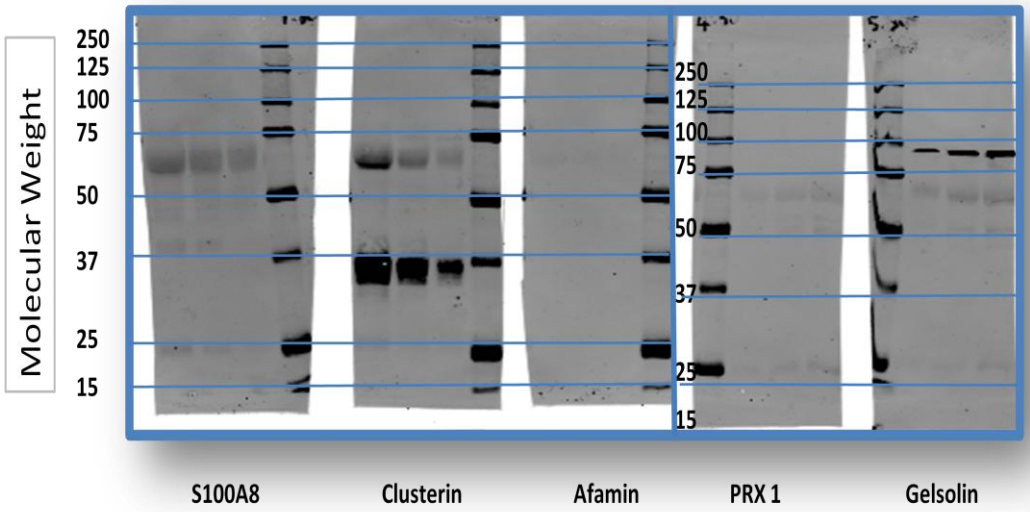

Figure 29. Western blots for the selected proteins of interest.

Table 34. Results of gelsolin quantification.

Gelsolin	N	Mean	SD	p	MD	SED	95% CI	
							Lower	Upper
Controls	87	0.44	0.15	.01**	0.52	0.20	0.11	0.09
Cases	125	0.39	0.13					

SD = Standard deviation; MD = Mean difference; SED = Standard error of the difference.
**Correlation is significant at the .01 level (2-tailed).

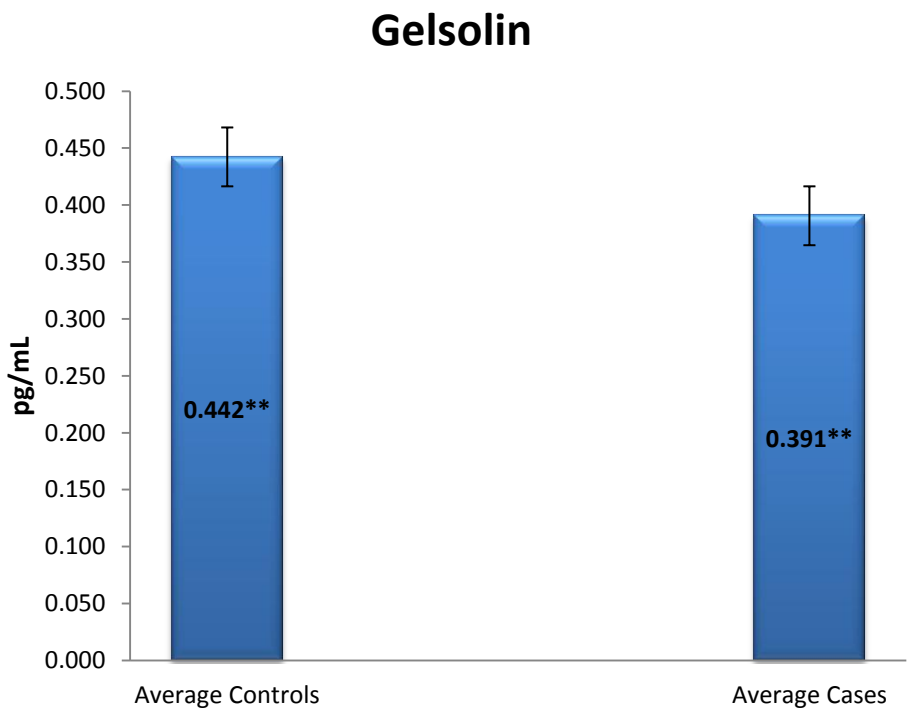

Figure 30. Gelsolin mean average values (p = .009).

** Correlation is significant at the .01 level (2-tailed).

S100A8 levels were also determined in the same validation sample. Student's t-test was used as the variable appeared to be normally distributed. Equal variances were not assumed following a non-significant result on the Levene's Test (0.457) and the p value was calculated at p = .9 (Table 35).

As no difference was obtained, no further validation on S100A8 was performed. Nonetheless, S100A8 is one protein of a family of 25, all of which are involved in Ca^{2+} regulation and thus, it was decided to test another protein from the same family, S100B, instead. To examine the expression levels of S100B we utilised the Millipore Human S100B ELISA 96-Well Plate (Cat. #EZHS100B-33K). The ELISA allowed the detection of S100B with excellent sensitivity with a mean CV of 7.35% between duplicates. The inter assay was calculated from the QC 1&2 across all plates (mean 9.2%). Standard curves were generated using a 5-parameter logistic (5-PL) regression fit (Sigma Plot, Sysstat Software Inc) with a linear range from S7-S5 for all analytes.

Quantified data from Sigma Plot was inputted into SPSS (v15), Shapiro-Wilk test for normality was performed ($p > .05$) indicating the data was not normally distributed. We therefore used nonparametric test (Mann–Whitney).

Comparison of the means showed a large increase in S100B levels in the schizophrenia group compared to the controls which was highly significant (p = .007) (Table 36 and Figure 31).

Table 35. Results of S100A8 quantification.

S100A8	N	Mean	SD	SED	p	MD	95% CI	
							Lower	Upper
Controls	58	0.68	0.13	0.02	.96	.00	-0.04	0.04
Cases	82	0.68	0.13	0.01				

SD = Standard deviation; MD = Mean difference; SED = Standard error of the difference.

Table 36. Results of S100B ELISA quantification in human plasma samples.

S100A8	N	Mean	SD	SED	p	MD	95% CI	
							Lower	Upper
Controls	75	56.49	40.65	4.69	.007**	-53.11	-91.61	-14.60
Cases	72	109.60	163.56	19.27				

SD = Standard deviation; MD = Mean difference; SED = Standard error of the difference. ** Correlation is significant at the .01 level (2-tailed).

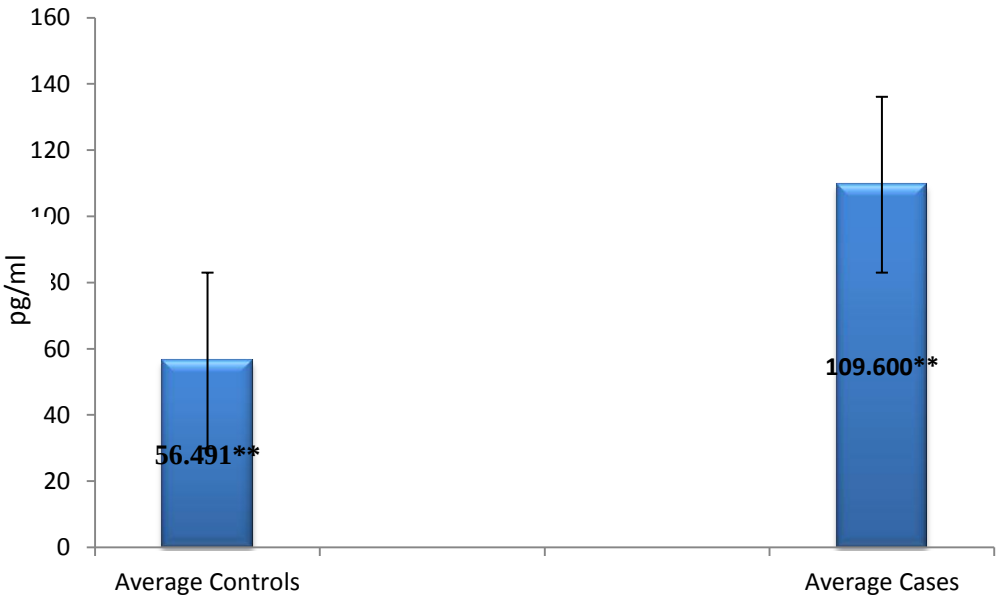

Figure 31. S100B average plasma levels (p = .007).

** Correlation is significant at the .01 level (2-tailed).

5.7. Summary

This study intended to examine whether classical proteomic approaches could be used to canvass the plasma of patients suffering from psychotic symptoms for disease biomarkers. Samples were collected, processed and stored in accordance with a standard operational protocol (SOP) (Hochstrasser *et al.* 1988) which has been optimised at the Lovestone lab and is routinely employed for biomarker studies in plasma in AD (Hye *et al.* 2006; Thambisetty *et al.* 2010b).

Fifteen subject pairs were selected from the GAP sample. Patients belonged to three treatment groups: (1) medication free; (2) treated with olanzapine; or (3) treated with risperidone. Controls were selected on the basis of being the closest match to the case for gender, age, ethnicity, smoking, alcohol and cannabis use.

Plasma was extracted from whole blood in EDTA coated tubes; samples were subjected to less than 3 freeze-thaw cycles prior to entering electrophoresis. Gels were obtained, scanned and analysed and the top eight spots yielding significant p-value and fold change following multiple corrections (FDR) were excised from MS-compatible gel matrixes and subjected to mass spectrometry. As a result, 14 proteins and potential targets were identified. Ten of these proteins were upregulated in cases and 4 in controls. From the first group S100A8, peroxiredoxin-1, clusterin and afamin were selected and from the second group gelsolin was carried forward for validation and examined using Western blots with commercial antibodies.

For two of those, gelsolin and S100A8, commercial antibodies were available. Gelsolin was found to be significantly lower in cases than in controls but no difference was observed in S100A8 expression levels between the groups. Failure to validate S100A8 was thought to be largely the result of the poor quality of the antibody. Hence, another protein of the same family, S100B was tested instead on the basis of 1) being a Ca^{2+} - binding protein of the same family; 2) previous reports of association with SCZ at proteomic (Lara *et al.* 2001; Petzold *et al.* 2003; Rothermundt *et al.* 2004; Schmitt *et al.* 2005; Zhang *et al.* 2010b) and genetic level (Liu *et al.* 2005; Roche *et al.* 2007); and 3) availability of an ELISA kit.

Gelsolin is a calcium-regulated protein that exerts its effect in the assembly and disassembly of actin filaments and maps to 9q33, a gene locus found to be implicated in modifying the clinical course and severity of SCZ (Fanous *et al.* 2007). Defects in this gene also cause familial amyloidosis finnish type (FAF), whereby systemic deposition of amyloid by the secreted (not cytoplasmic) form of the protein is found in most tissues resulting in a characteristic triad of ophthalmological, neurologic and dermatological symptoms (Kiuru,

1992). A recent study form the Lovestone group has shown that AD patients also displayed significantly lower plasma gelsolin levels compared to non-denmented controls (NDC) subjects and correlated with disease progression rate estimated by Mini-Mental Status Examination (Guntert *et al.* 2010).

S100 proteins are Ca^{2+} - binding proteins of small molecular weight (10-12 KDa) which are located in chromosomes 1q21 (S100A1-A18, trychohylin, fillagranin, repetin), 21q22 (S100B), Xp22 (S100G), 4p16 (S100P) and 5q14 (S100Z) and are present exclusively in vertebrates (Sedaghat and Notopoulos, 2008). S100B is the most abundant member of the S100 family expressed in the brain, predominantly synthesized and secreted by astrocytes. In addition, S100B, maps to 22q21, an area of linkage for schizophrenia and BPAD with psychosis (Liu *et al.* 2009; Roche *et al.* 2007) which has been identified as regulatory for presynaptic calcium signalling and synaptic plasticity in a mouse model of 22q11 deletion syndrome (Earls *et al.* 2010).

Increased extracellular levels of S100B in the brain are indicative of neuronal injury or underlying chronic inflammation that can be subsequently measured in the peripheral circulation, after passing through the blood brain barrier. S100B, has previously been associated with SCZ in CSF (Rothermundt *et al.* 2004), and found to be increased in medication-free patients with a negative correlation with illness duration, indicating that a neurodegenerative process may take place in a subset of people suffering from SCZ (Lara *et al.* 2001). In this study, we have used an ELISA that was designed to measure S100B in plasma, serum and CSF.

The main limitations of this study are two fold. Firstly, 2DPAGE is labour intensive, quantification is poor, automation is difficult and cannot be coupled directly to mass spectrometry (Wang *et al.* 2002). Secondly, in this study only one protein (gelsolin) resulting from the discovery phase was validated. Not all the commercial antibodies obtained were shown to work on plasma and a signal at the predicted height corresponding to the theoretical molecular weight was identified in only two of them. Here, we present a retrospective study of samples collected from patients experiencing psychotic symptoms along with age matched controls on the levels of gelsolin and S100B found in the peripheral circulation. We have showed that classic proteomic techniques can be used for SCZ biomarker research in plasma and bear great promise for discovery of altered signalling pathways and molecular processes.

The results of this study suggest that a subset of people presenting with psychotic symptoms might be suffering from underlying altered calcium signaling pathways.

CHAPTER 6: General discussion

6.1. Schizophrenia plasma proteomics

The search for biomarkers in plasma in SCZ research started well over 50 years ago with the study of the variation in plasma aminoacids, serum lipids and proteins during psychotic episodes and periods of recovery (Wang *et al.* 2002) as well as following prefrontal lobotomy (Man *et al.* 1947a). Since then, proteomic studies have been conducted using serum, plasma, or cellular fractions of blood. Proteins for which two or more studies have been conducted and the changes in protein expression appear to be concordant and/or have been identified in plasma and in at least one other different tissue (brain, CSF, or serum) in schizophrenia are:

1. Brain derived neurotrophic factor (BDNF)

BDNF has been found downregulated in plasma in two studies in SCZ (Palomino *et al.* 2006; Tan *et al.* 2005) and in the plasma and CSF of drug-naïve first-episode psychotic patients (Issa *et al.* 2010; Pillai *et al.* 2010). In addition, it was reduced in the serum (Cunha *et al.* 2006) and plasma (Palomino *et al.* 2006) of bipolar patients. On the other hand, it has been shown to be upregulated in serum in SCZ in one other study (Jockers-Scherubl *et al.* 2004). BDNF promotes the survival and differentiation of selected neuronal populations of the peripheral and central nervous systems during neurodevelopment, participates in axonal growth, pathfinding, and in the modulation of dendritic growth and morphology. BDNF regulates synaptic transmission and plasticity at adult synapses and contributes to long-term potentiation (LTP), long-term depression (LTD) and to the homeostatic regulation of intrinsic neuronal excitability. Defects in BDNF are a cause of congenital central hypoventilation syndrome (CCHS), a rare disorder characterized by abnormal control of respiration.

2. Neural cell adhesion molecule 1 (NCAM1)

NCAM serum levels have been shown to be raised in plasma (Lyons *et al.* 1988) and in CSF of SCZ patients (Poltorak *et al.* 1997; Vawter *et al.* 2001) and also in the plasma of AD patients (Todaro *et al.* 2004). It is a cell adhesion molecule involved in cell-to-cell interactions as well as cell-matrix interactions during development and differentiation and also in neurite fasciculation and outgrowth. NCAM1 is involved in development of the nervous system, and in the expansion of T cells and dendritic cells, which play an important role in the immune surveillance of the CNS.

3. Transthyretin (TTR)

TTR was downregulated in plasma of SCZ patients in two studies (Wan *et al.* 2006; Yang *et al.* 2006) and in CSF of SCZ patients (Huang *et al.* 2006a; Wan *et al.* 2006). It was also reduced in the serum of patients with AD (Elovaara *et al.* 1986). TTR probably transports thyroxine from the bloodstream to the brain. TTR defects are a cause of (1) hyperthyroxinemia

dystransthyretinemic euthyroidal (HTDE), a condition characterized by raised levels of total and free thyroxine in euthyroid subjects and (2) carpal tunnel syndrome type 1.

4. Nerve Growth Factor (NGF)

NGF expression has been found to be downregulated in plasma, serum, and in CSF of SCZ patients (Kale *et al.* 2009; Xiong *et al.* 2010), in plasma of BPAD patients (Barbosa *et al.* 2010), and in serum of PD patients (Lorigados *et al.* 2002). NGF is important for the development and maintenance of the sympathetic and sensory nervous systems. It stimulates division and differentiation of sympathetic and embryonic sensory neurons. Defects in NGF are the cause of hereditary sensory and autonomic neuropathy type 5 (HSAN5). HSAN5 patients manifest loss of pain perception and impaired temperature sensitivity, ulcers, and in some cases self-mutilation.

5. Interleukin-2 (IL2)

IL2 expression was upregulated in the plasma and serum of patients with SCZ (Ganguli and Rabin, 1989; Maes *et al.* 1995b), and in their CSF (Licinio *et al.* 1993; McAllister *et al.* 1995), in the plasma of BPAD patients (Maes *et al.* 1995a), in serum of PK patients (Brodacki *et al.* 2008) and in mononuclear cells of AD patients (Huberman *et al.* 1994). Reduced levels were found in peripheral blood mononuclear cells (PBMC) of PD (Bessler *et al.* 1999), and AD patients (Beloosesky *et al.* 2002). IL2 is type-1 secreted cytokine involved in the proliferation of lymphocites T and B.

6. Interleukin-6 (IL6)

IL6 levels were upregulated in the serum of SCZ patients according to 2 studies (Maes *et al.* 1995a; Naudin *et al.* 1996) and in the CSF of patients with SCZ (Garver *et al.* 2003; van Kammen *et al.* 1999). The expression of its receptor (IL6R) was also upregulated according to another one (Muller *et al.* 1997). IL6 has also been found upregulated in the plasma of BPAD (Maes *et al.* 1995a) and AD patients (Swardfager *et al.* 2010). IL6 is a pro-inflammatory cytokine (type-1) which has been shown to be an endogenous pyrogen. The IL6 gene is implicated in a wide variety of inflammation-associated disease states including susceptibility to diabetes and systemic juvenile rheumathoid arthritis.

These data suggest alterations in plasma in schizophrenia indicative of disfunction in the regulation of synaptic transmission, nervous system development and thyroid function. In addition, they may have sensory and autonomic deficits (loss of pain perception and impaired temperature sensitivity), susceptibility to diabetes and alterations in their immune response.

In a recent paper, a serum protein signature of 34 analytes was identified in a cohort of closely matched first onset SCZ patients (n = 71) and controls (n = 59) (Schwarz *et al.* 2011a). These analytes were: α-1-antitrypsin (a1AT), α-2-macroglobulin (A2M), angiopoietin 2 (ANG2), BDNF, betacellulin, bone morphogenic protein (BMP6), carcinoembryonic antigen (CEA), CD40 ligand (CD40L), cortisol, connective tissue growth factr (CTGF), epidermal growth factor (EGF), eotaxin 3, factor VII, follicle stimulating hormone (FSH), granulocyte macrophage colony stimulating factor (GM-CSF), gluthathione S transferase (GST), haptoglobin (HPT), intercellular adhesion mollecule 1 (ICAM 1), insulin-like growth factor binding protein (IGFBP 2), IL10, IL17, IL5, luteinizing hormone (LH), macrophage migration inhibitory factor (MIF), NCAM, pancreatic polypeptide (PP), prostatic acid phosphatise (PAP), RANTES (C-C motif chemokine 5), resistin, serum glutamic oxaloacetic transaminase (SGOT), sortilin, stem cell factor (SCF), thrombopoietin (TPO), and thrombospondin 1 (TSP1).

Most of these proteins are involved in immunoregulation, cardiovascular diseases, and metabolic alterations, and in cellular migration and proliferation. Caution should be exercised when interpreting these results, as some of them, like haptoglobin and α-1-macroglobulin were shown in Chapter 4 of this thesis to be upregulated by haloperidol and citalopram in plasma, whereas glutathione S transferase was downregulated in the striatum of the F344 rats.

6.2. Summary of my findings

6.2.1. Examining the effects of DISC1 knockdown on the proteome of human progenitor cells

The DISC1 affected Scottish pedigree included 23 members who met Research Diagnostic Criteria (RDC) for mental disorders, out of which 3 were cases of SCZ, 2 of schizoaffective disorder, 6 of major depressive disorder, 3 of generalised anxiety disorder, 1 of minor depressive disorder and 3 of alcoholism. There were also 3 who attempted suicide and 2 who completed it. A further 5 members were being treated for severe conduct disorder, learning disabilities, bizarre behaviour, and emotional disorders (St *et al.* 1990) . Since then, DISC1 has been associated with chronic fatigue syndrome (Fukuda *et al.* 2010), autism and mental retardation (Crepel *et al.* 2010), Asperger syndrome (Kilpinen *et al.* 2008), depression (Hashimoto *et al.* 2006), and bipolar disorder (Maeda *et al.* 2006).

The approach used in this study to examine the proteome in SCZ is a novel discovery approach. A mimicked haploinsufficiency of DISC1 in human neural progenitor cells was investigated at a protein expression level. DISC1 expression was reduced in both DISC1 RNAi treated groups (35% reduction with RNAi1 and a 38% reduction for the samples treated with

RNAi2). Analysis of the cellular proteome was carried out by using 2DPAGE coupled to LC/MS/MS and two spots containing one single analyte each, were identified in this study as being regulated by both RNAi groups with a statistically significant difference with the control RNAi group. Dihydropteridine reductase (DHPR) was downregulated and peptidyl-prolyl isomerase A (cyclophilin A) was upregulated by both RNAi treatment groups.

The DHPR gene locates to 4p15.31. Deletions in this area have been associated to development of cervical cancer (Singh *et al.* 2007), basal-like breast tumours (Horlings *et al.* 2010), lung cancer (Davidson *et al.* 2010) and bladder cancer (Tatarano *et al.* 2011). In neuropsychiatric disorders, comparative gains in copy number variations have been found in Parkinson's disease (Kim *et al.* 2008). DHPR reached gene-wide significance in bipolar affective disorder (Shi *et al.* 2008) but failed to survive correction for multiple testing. In addition, a SNP located on 4p15.31, was associated with a reduced risk of increasing suicidal ideation during treatment with escitalopram (Perroud *et al.* 2010).

DHPR can be found in cytoplasm, cytosol and mitochondrion. It catalyzes the NADH-mediated reduction of quinonoid dihydrobiopterin. The product of this enzyme, tetrahydrobiopterin (BH4), is an essential cofactor for phenylalanine, tyrosine, and tryptophan hydroxylases and is therefore important in the biosynthesis of dopamine and serotonin (Figure 32).

A marker of DHPR deficiency, which leads to tetrahydrobiopterin or (BH4) deficiency and impaired biogenic amines synthesis, is neonatal hyperphenilanemia. BH4 is an essential cofactor of aminoacid hydroxylases which are the rate-limiting enzymes in phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) catabolism and in biosynthesis of the neurotransmitters dopamine and serotonin. BH4 enhances the release of dopamine and serotonin in neurons (Liang and Kaufman, 1998) and is a neuroprotective factor via the nitric oxide (NO) synthase pathway (Thony *et al.* 2000). BH4 deficiencies have been associated with Alzheimer's disease, Parkinson's disease, autism, and depression (Thony *et al.* 2000).

More recently, DHPR was found to be upregulated in the Wernicke's area (Martins-De-Souza *et al.* 2009a) and in the thalamus and CSF (Martins-De-Souza *et al.* 2010) of SCZ patients. On the other hand, DHPR activity has been measured in blood in a small sample of people with SCZ and healthy controls but no differences were found between the groups (Szymanski *et al.* 1985).

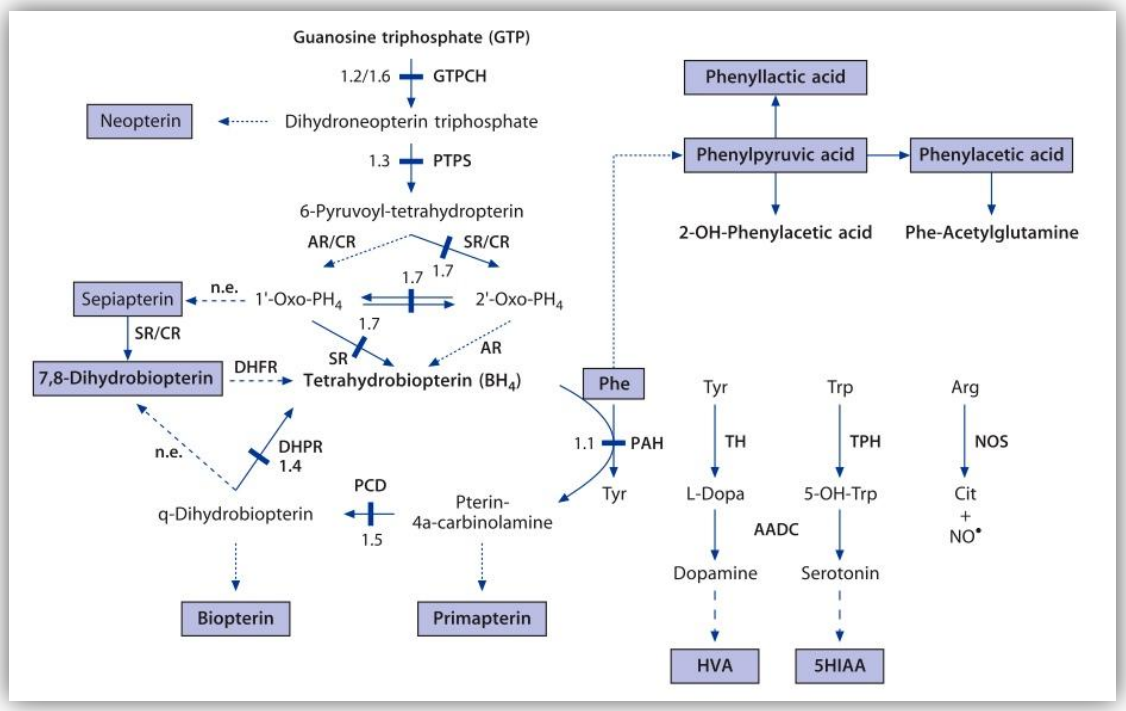

Figure 32. Biosynthesis and regeneration of tetrahydrobiopterin including possible metabolic defects and catabolism of phenylalanine.

1.1 = phenylalanine-4-hydroxylase (PAH); 1.2/1.6 = GTP cyclohydrolase I (GTPCH), 1.3 = 6-pyruvoyl-tetrahydropterin synthase (PTPS), 1.4 = dihydropteridine reductase (DHPR), 1.5 = pterin-4-a-carbinolamine dehydratase (PCD), 1.7 = sepiapterin reductase (SR), carbonyl reductase (CR), aldolase reductase (AR), dihydrofolate reductase (DHFR), aromatic aminoacid decarboxylase (AADC), tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH), nitric oxide synthase (NOS).

The cyclophilin A (PPIA) gene maps to chromosomic region 7p13 and has been weakly linked to mood-incongruent psychosis in bipolar affective disorder (Hamshere *et al.* 2009). Cyclophilin A (PPIA) catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and accelerate the folding of proteins.

Cyclophilin A is a proinflammatory cytokine that activates endothelial cells and is the main target of cyclosporine A (Jin *et al.* 2004). PPIA is secreted from smooth muscle and macrophages in response to oxidative stress (Jin *et al.* 2004). This protein is a cyclosporin binding-protein and may play a role in cyclosporin A-mediated immunosuppression. The complex cyclosporin-PPIA inhibits calcineurin, a calcium/calmodulin dependent phosphatase. The protein can also interact with several HIV proteins, including p55 gag, Vpr, and capsid protein, and has been shown to be necessary for the formation of infectious HIV virions, regulating HIV-1 and hepatitis C infectivity (Braaten and Luban, 2001; Liu *et al.* 2009).

Cyclophilin A is upregulated in endometrial carcinoma (Li *et al.* 2008) and is involved in the progression of human breast cancer (Zheng *et al.* 2008), being necessary for the prolactin (PRL)-induced activation of the Janus-activated kinase 2 tyrosine kinase (Jak2/STAT) signaling pathway by interacting with the human prolactin receptor (Syed *et al.* 2003). Cyclophilin A is upregulated in small cell lung cancer and activates ERK1/2 signaling (Yang *et al.* 2007). In neuropsychiatric disorders, the role of PPIA is not yet fully understood. It appears that it has a role in apoptosis as it augments chromatinolysis synergically with apoptosis inducing factor (AIF) (Cande *et al.* 2004), which moves from mitochondria to nuclei following cerebral hypoxia-ischemia (Zhu *et al.* 2007). A recent study showed that PPIA was downregulated in the anterior cingulate cortex of schizophrenic males (Martins-De-Souza *et al.* 2010) but was found in a different study by the same authors to be upregulated in the thalamus and CSF of SCZ patients (Martins-De-Souza *et al.* 2010) , which may suggest a region-specific profile expression. In addition, PPIA appears to promote atherosclerosis in apolipoprotein-E deficient mice (Nigro *et al.* 2011).

In this study, a classical proteomic approach has been used to show that DISC1 haploinsuficiency results in downregualtion of DHPR and thus, alteration in the synthesis of dopamine and serotonin as well as upregulation of PPIA. These alterations are likely to act synergistically inducing the molecular alterations responsible for the emergence of psychopathology in terms of affective and psychotic symptoms, as well as some of the cytostructural alterations frequently observed in people with schizophrenia such as reduced overall brain weight, thinning of grey matter in the absence of gliosis and dysfunctional apoptosis.

6.2.2. Examining the effects on brain and blood proteome of antipsychotic and antidepressant medication on F344 inbred rats

A classical proteomic approach was used to examine two target tissues (plasma and striatum) as well as the control tissue (prefrontal) from F344 inbred rats treated with haloperidol, citalopram and a control (vehicle) solution through surgically implanted osmotic mini-pumps. Serotonin levels were measured in plasma and prefrontal cortex to verify that medication reached target organs and 2DPAGE coupled to LC/MS/MS was used to examine differential proten expression in plasma and striatum. Following analysis of the gels using Progenesis SameSpots software, 19 spots from plasma and 18 from striatum were selected on the bases of their p-value, fold change and FDR.

Twenty-two different proteins were identified in 19 plasma spots: α-1-antiproteinase; α-1-macroglobulin; angiotensinogen; anionic trypsin-2; apolipoprotein A-IV; ATP synthase subunit beta, mitochondrial; beta-2-glycoprotein 1; carbonic anhydrase 1; carbonic anhydrase 2; complement C3; fibrinogen α chain; fibrinogen beta chain; fibrinogen gamma chain; haptoglobin; Ig gamma-2B chain C region; Ig kappa chain C region, A allele; Ig lambda-2 chain C region; plasminogen; pyruvate kinase isozymes M1/M2; serotransferrin; tubulin α-1C chain and vitamin D-binding protein.

And 30 in 18 striatum spots: acyl-protein thioesterase 1; aspartate aminotransferase, cytoplasmic ; ATP synthase subunit α, mitochondrial ; carbonic anhydrase 2; creatine kinase U-type, mitochondrial; dihydropteridine reductase; dihydropyrimidinase-related protein 2; fructose-bisphosphate aldolase A; fructose-bisphosphate aldolase C; glutamine synthetase; glutathione S-transferase P; glyceraldehyde-3-phosphate dehydrogenase; isocitrate dehydrogenase [NAD] subunit beta, mitochondrial ; malate dehydrogenase, mitochondrial; NAD-dependent deacetylase sirtuin-2; NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial; phosphoglycerate kinase 1; phosphoglycerate mutase 1; protein DJ-1; pyruvate dehydrogenase E1 component subunit α, somatic form, mitochondrial; pyruvate kinase isozymes M1/M2; Rho GDP-dissociation inhibitor 1; succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial; triosephosphate isomerase; tubulin beta-2C chain; ubiquitin carboxyl-terminal hydrolase isozyme L1; voltage-dependent anion-selective channel protein 1; voltage-dependent anion-selective channel protein 2; V-type proton ATPase subunit B, brain isoforms and V-type proton ATPase subunit E1.

A list of upregulated proteins and another one of downregulated proteins was prepared and inputed to generate biological networks. A system biology analysis was

performed using GeneGo (www.genego.com). GeneGo provided data mining analysis to identify and prioritise the most relevant pathways, networks and cellular processes altered by the effect of the drug. The main parameters for the analysis were (a) relative enrichment with the uploaded data, and (b) relative saturation of networks with canonical pathways. Networks were then prioritized based on the number of fragments of canonical pathways present on them.

Three proteins were present in both lists and were mostly downregulated in plasma and striatum by haloperidol and citalopram: carbonic anhydrase 2, pyruvate kinase isozymes M1/M2 and tissue-specific chains of tubulin, in plasma (alpha-1C) and in brain (beta-2C).

Carbonic anhydrase (CAH2) was downregulated in both treatment groups in plasma and in striatum. CAH2 is an enzyme with carbonate dehydratase activity ($H_2CO_3 = CO_2 + H_2O$) and metal, protein and zinc ion binding properties. It is involved in carbon dioxide transport, response to pH, response to stress, response to steroid hormone stimulus, regulation of bone resorption, odontogenesis and kidney development mainly. It is extremely ubiquitous and can be found in the apical part of the cell, the axon, the basolateral plasma membrane, the cytoplasm and cytosol, the extracellular space, the microvillus and the nucleus. CAH2 is involved in osteoporosis and its transcription is regulated (activated) by the vitamin D3 Receptor (VDR) in monocytic cells (Quelo and Jurdic, 2000).

Its Homo sapiens homolog is a 29246 Da protein coded by the CA2 gene, located in 8q21.3-q22, and partakes in similar cell signalling pathways and molecular processes. This chromosomal region has been recently shown to have a weak linkage to SCZ (Wiener *et al.* 2009) and a possible association with juvenile bipolar disorder phenotype (Doyle *et al.* 2010). Evidence of alteration in this protein in SCZ has been suggested by Martins-De-Souza and colleagues recently (Martins-De-Souza *et al.* 2009b), who found it to be down regulated in the anterior temporal lobe of post-mortem samples of SCZ patients. Nonetheless, given the cellular mechanisms this protein is involved in and the fact that it was downregulated as a result of medication in an animal model of healthy rodents, it is probably safer to assume that the finding of Martins-de-Souza and colleagues was due to the effect of medication rather than being part of the etiopathological process of the disorder.

A second protein that appeared in both lists was the mitochondrial pyruvate kinase isozymes M1/M2 (KPYM_RAT). It was downregulated in both plasma and brain in the haloperidol treated group and in the striatum of the citalopram treated group, whereas it was upregulated in the plasma of the citalopram treated animals. KPYM_RAT is a glycolytic enzyme located in the cytoplasm, cytosol, flagellum, mitochondrion, nucleus and plasma membrane of the cell as well as having a soluble fraction. It catalyzes the transfer of a phosphoryl group from

phosphoenolpyruvate (PEP) to ADP, generating ATP. It is involved in ATP metabolism, G-protein signalling, glycolisis and gluconeogenesis and pyruvate metabolism (Sugden and Holness, 2002). It participates in insulin signal transduction and potassium ion transport. It is also involved in the organism response to hypoxia, liver development and programmed cell death (Kietzmann *et al.* 2002).

The human homolog of this protein, KPYM maps to gene PKM2, located on 15q22 which has been shown to be an area of linkage in SCZ (Stone *et al.* 2004). Moreover, a decrease in the levels of pyruvate, the final product of glycolisis, has been previously reported in the mediodorsal thalamus but not in the CSF of SCZ patients (Martins-De-Souza *et al.* 2010). Whether this alteration is a medication effect or integral part of the pathophysiology of SCZ remains to be determined. The expression of the enzyme is induced by insulin mediated activation of PI3K and MAPK signalling pathways (Mazurek, 2011). Activation of PI3K/Akt signalling promotes phosphorylation/inactivation of GSK-3, suppresses GSK-3-dependent phosphorylation of tau at residues overphosphorilated in AD and prevents apoptosis of confluent cells and may prevent development of AD pathology (Baki *et al.* 2004). Downregulation of PKM2 may thus alter GSK3-beta signalling which has been speculated to be altered in SCZ patients (Souza *et al.* 2008).

Lastly, the third altered protein, tubulin, is involved in microtubule-based movement and protein polymerization and is an integral component of cytoplasm, cytosol, microtubules and cytoskeleton. It plays a crucial role in cell division, vesicle formation and exosomal release, as well as in maintaining effective intracellular transport of organelles, such as mitochondria from the soma to the synapse, and in stabilising the synapse by establishing intersynaptic filaments that attach the synaptic bulbs from the axon of the presynaptic neuron to the plasma membrane of the opposing neuron. This dimer of alpha and beta chains, had its α-1C chain (Tuba1c) downregulated in plasma in both treatment groups, and its beta-2C chain (Tubb2c), which is expressed exclusively in neurons, was downregulated as well in both groups relative to vehicle treated animals in the striatum samples.

Tuba1c (7q36) has nucleotide binding activity and GTPase activity. The homolog human gene is TUBA1C, located in 12q13.12; a gene associated with human lissencephaly and cerebellar hypoplasia (Lecourtois *et al.* 2010) and thus, suspected of playing a crucial role in neurodevelopment. TUBA1C was identified as a unique candidate DTNBP1-interacting protein in HEK293 human cell line using SDS polyacrylamide gel electrophoresis and HPLC/MS/MS recently (Mead *et al.* 2010), suggesting that impairments in vesicle formation could be part of a pathogenic mechanism in SCZ.

Tubb2c (3p13) has similar activity, molecular function and structural roles to Tuba1c. The human homolog gen, TUBB2C, is located in 9q34, an area recently linked to SCZ which also contains the dopamine β-hydroxylase (DβH) gene (DBH) (Cubells *et al.* 2011).

In addition, deletions of the TUBA1C gene have been linked to oestrogen receptor negativity in breast cancer patients (Pierga *et al.* 2007). Tubulins are known targets for 'vinca alkaloid' drugs such as vinblastine and vincristine in oncology and also for colchicine, an anti-gout agent that binds to tubulin, inhibiting microtubule formation and neutrophil motility, reducing inflammation. The observed haloperidol-induced downregulation of tubulin may have similar effects to these agents. In fact, an antiproliferative effect of a haloperidol metabolite has been recently reported in prostate cancer cells (Marrazzo *et al.* 2011).

GeneGo (www.genego.com) was used to examine the pathways likely to be altered by haloperidol and citalopram using the candidate protein lists yielded by the Mann-Whitney tests. In order to simplify the network analysis, only the first two top candidates per spot were used as these were considered to be the ones more likely to be accountable for the differences in expression observed in the individual spots. Networks were then prioritized based on the number of fragments of canonical pathways present on the network.

The main networks altered by haloperidol in plasma were:

1. Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway
2. Angiotensin II, fibrinogen gamma, iC3b, fibrinogen α, C3a
3. C3, PZP (pregnancy zone protein or α-1-macroglobulin), fibrinogen (fibrin), angiotensinogen, fibrinogen beta.
4. Angiotensin (1-7), MAS1.
5. Alpha 1-antitrypsin, PZP (pregnancy zone protein or α-1-macroglobulin), kallikrein 12, kallikrein 3 (prostate specific antigen, PSA), kallikrein 5.

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway or cascade is involved in growth hormone signaling pathway, response to wounding and positive regulation of response to external stimulus. JAK activation stimulates cell proliferation, differentiation, migration and apoptosis. This pathway was suppressed in plasma by the effects of haloperidol.

Angiotensin II is an inhibitory factor of PRL secretion (Leite *et al.* 2008), and the downregulation of this inhibition may mediate dopamine regulated hyperprolactinemia amongst other things.

Alpha-1-macroglobulin is a protease inhibitor that acts as regulator of neuronal development and function. Its active form depresses dopaminergic and cholinergic transmission (Hu *et al.* 1998). Hence, an excess of dopaminergic transmission could be the result of a deficit in the active form of this protein. The downregulation of this pathway by haloperidol may be suggestive of a non-synaptic therapeutic effect of the medication or of a primary defect in its regulation, which may be central to the appearance of psychotic disorders.

MAS1 is the receptor for angiotensin 1-7 and when activated, it modulates a critical component in a growth-regulating pathway to bring about oncogenic effects. It is also involved in maintaining blood pressure and in intracellular signaling probably through Akt phosphorylation, protein kinase C activation and mitogen-activated protein (MAP) kinase inhibition (Iwai and Horiuchi, 2009). The downregulation of this pathway by haloperidol in plasma is another example of its antiproliferative activity (Marrazzo *et al.* 2011), which has only recently started to be ascertained, and may be mediated by its antagonism to the σ receptor subtype (Vilner *et al.* 1995).

In the striatum, the following signalling pathways where mainly altered by haloperidol:

1. CRMP2 (collapsing response mediator protein 2), ATP5A (ATP synthase subunit α, mitochondrial), succinate dehydrogenase, MPTP (mitochondrial permeability transition pore) complex, SDHA (Succinate dehydrogenase subunit A).
2. VDAC 1 (voltage-dependent anion-selective channel protein 1), PKM2 (pyruvate kinase isozyme M1/M2), VDAC 2, DHPR (Dihydropteridine reductase), TPI1.
3. G3P2 (glyceraldehyde-3-phosphate dehydrogenase), DJ-1 (Parkinson disease autosomal recessive, early onset 7, PARK7), CAR-2 (carbonic anhydrase II), VDAC 1, p53.
4. DJ-1, G3P2 (glyceraldehyde $3PO_4$ dehydrogenase), androgen receptor, p53, c-Myc.

CRMP2 (also known as dihydropyrimidinase related protein, DRP) plays a critical role in axon outgrowth and axon-dendrite specification (Uchida *et al.* 2005). It is a physiological target of GSK-3-beta (Soutar *et al.* 2010). This signaling pathway was shown to be downregulated by haloperidol in this thesis. A recent study examining the effects of antipsychotics (haloperidol and clozapine), mood stabilisers (lithium and valproic acid) and antidepressants (imipramine and fluoxetine) on GSK-3, Akt and Wnt in the rat prefrontal cortex and striatum found that only antipsychotic treatment regulated Wnt pathway and enhanced the association of GSK-3 at the dopamine D2 receptor (Sutton and Rushlow, 2011).

ATP5A is the gene coding for the subunit of the soluble catalytic core (F1) of mitochondrial ATP synthase. The other component (F0) is a membrane-spanning proton channel. During oxidative phosphorylation mitochondrial ATP synthase produces ATP from ADP in the presence of a proton gradient across the inner membrane. It appears to be upregulated by haloperidol, which may explain the therapeutic action on decreased oxidative metabolism found in SCZ brains (Benitez-King *et al.* 2010) but be equally responsible for the neuronal damage observed following chronic treatment, leading to extrapyramidal symptoms and neurocytoskeletal disorganization (Benitez-King *et al.* 2010).

Succinate dehydrogenase (SDH) is a crucial enzyme of the Krebs cycle as an electron transferring protein in the mitochondrial respiratory chain where most cell energy is obtained through oxidative phosphorylation. Consistent with previous findings (Streck *et al.* 2007), it was found to be downregulated in this study, suggesting that long term treatment with haloperidol worsens a pre-existing pathological state of deficient cell energy generation. This may explain the appearance of cognitive dysfunction, negative symptoms and extrapyramidal side-effects known to be associated with the chronic administration of typical antipsychotics. The MPTP (mitochondrial permeability transition pore) complex is a protein pore formed by multiple macromolecular components (voltage-dependent anion-selective channel proteins 1 and 2, amongst others) in the membranes of mitochondria under certain conditions such as traumatic brain injury or stroke and that can lead to mitochondrial swelling and subsequent cell death.

TPI1 is an enzyme on the glycolisis/gluconeogenesis pathway that catalyzes the isomerisation of glyceraldehyde 3-phosphate (G3P) and dihydroxy-acetone phosphate (DHAP). It was downregulated by haloperidol, consistent with the notion of iatrogenic decrease in cell energy production and has recently been shown to be increased by clozapine (Baig *et al.* 2010), raising the speculation that this pathway might explain the metabolic syndrome seen patients treated with atypical antipsychotics.

Pathway 3 appears entirely downregulated by haloperidol. G3P2 catalyzes the sixth step of glycolisis, adding up evidence to the detrimental effect haloperidol exerts on cellular energy metabolism. DJ-1 is a neuroprotective transcriptional co-activator, which reduced expression by haloperidol leaves dopaminergic neurons vulnerable to apoptosis and may contribute to the emergence of extrapyramidal symptoms (Xu *et al.* 2005). Carbonic anhydrase II is a metalloenzyme that catalyzes the reaction of CO_2 and H_2O to HCO_3^- and H^+ and vice-versa and is stimulated by Vitamin D3. A downregulation on this enzyme will have a similar effect to carbonic anhydrase inhibitors such as acetazolamide, dorzolamide and topiramate which are used as antiglaucoma agents, antidiuretics, antiepileptics, and in the

management of altitude sickness. In addition, lack of carbonic anhydrase result in a reduction of hydrogen ions causing increased levels of free radicals leading ultimately to cell death.

Pathway 4, downregulated by haloperidol in striatum, contains p53, a tumour suppressor protein activated by Akt1, and c-Myc, a transcription factor that inhibits Akt1. Haloperidol downregulation of these proteins alters the cell cycle leading to apoptosis and supports its antiproliferative activity in cancer cells (Marrazzo *et al.* 2011).

Citalopram affected mainly the following networks in plasma:

1. C3, fibrinogen, fibrinogen gamma, fibrinogen α, iC3b.
2. ATP5B (ATP synthase subunit beta, mitochondrial), TCR (T cell (lymphocyte) receptor) gamma/delta.
3. CAR, CXADR (coxsachie virus and adenovirus receptor), CAR/RXR-α (androstane receptor (CAR, NR1I3)/retinoid X receptor α), ESR1 (nuclear), c-Jun.
4. CAR/RXR- α, PKM2 (pyruvate kinase isoenzymes M1/M2), PZP, ESR1 (nuclear), c-Jun.
5. PZP, C3a, c-Jun, c-Src (proto-oncogen tyrosine-protein kinase Src), annexin II.
6. CAR, MKK7 (MAP2K7, Mitogen activated protein kinase kinase 7), PNRC2 (proline-rich nuclear receptor coactivator 2), RAR (retinoic acid receptor), Ephrin-B receptors.

The observed upregulation of proteins in pathway 1, most of which are involved in blood coagulation, hemostasis, wound healing and regulation of body fluid levels, may explain the weak effect favouring abnormal hemostasis observed in patients treated with SSRIs (Alderman *et al.* 1996; Layton *et al.* 2001). Alternative explanations for SSRI-induced bleeding have been attributed to excess secretion of gastric acid and offered elsewhere (Andrade *et al.* 2010).

Alterations in pathway 2 indicate abnormal lipid metabolism as a result of citalopram treatment, resulting in release of glycerol and free fatty acids into the blood stream. ATP5B is the gene that codes mitochondrial ATP synthase subunit beta, which is responsible for catalyzing ATP synthesis during oxidative phosphorylation. TCR gamma/delta is a surface receptor present in a subset of lymphocyte T cells located in the gut mucosa which are thought to be involved in the recognition of lipid epitopes. These findings are consistent with previous reports of citalopram-induced generalized lipidosis in rats (Lullmann-Rauch and Nassberger, 1983) and phospholipidosis in U937 cells (Hutchinson *et al.* 2008), adding strength to the potential perturbed lipid metabolism observed in citalopram treated subjects (Flechtner-Mors *et al.* 2008).

The human gene CXADR codes a cell adhesion protein called coxsackievirus and adenovirus receptor (CAR) which is expressed during development in the CNS. The human constitutive androstane receptor/retinoid x receptor α (*CAR/RXR-α)* regulates transcription of target genes and modulates cytochrome P-450 genes function (Honkakoski *et al.* 1998), variations of which have been reported to be associated with tolerance and remission in patients treated with citalopram (Mrazek *et al.* 2011). C-Jun forms the early response transcription factor AP-1 with c-Fos, which has been shown to be altered by citalopram previously (Jensen *et al.* 1999), and to be responsible for modulation of HPA axis activity.

SMADs are intracellular proteins that act as transcription factors regulating the expression of certain genes by activating downstream transforming growth factor beta (TGF-β) gene transcription which is involved in cell growth, differentiation, apoptosis and cellular homeostasis. These are also the functions in which PKM2 and c-Jun are involved in, suggesting a role of citalopram in apoptosis. This role has already been reported as SSRI-induced apoptosis on Burkitt lymphoma cells (Serafeim *et al.* 2003). C-Jun, c-Src and Annexin II are linked by the S100 pathway. S100A6 interacts with Annexin 2 (Nedjadi *et al.* 2009), S100A7 with c-Jun (Emberley *et al.* 2005) and c-Src with S100A4 (Watanabe *et al.* 1993); which indicates an effect of citalopram in calcium-regulated processes, such as vesicle generation and exocytosis, microfilament assembly, cardiac and skeletal muscle signalling, for instance.

Mitogen activated protein kinase kinase 7 (MKK7, MAP2K7) is a kinase involved in mediating cell response to proinflammatory cytokines and regulates JNK signalling. Proline-rich nuclear receptor coactivator 2 (PNRC2) modulates transcriptional activation of nuclear receptors including estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR), thyroid hormone receptor (TR), retinoic acid receptor (RAR), and retinoid X receptor (RXR) in a ligand-dependent manner (Zhou and Chen, 2001). Ephrin (Eph) receptors are the largest subfamily of receptor tyrosine kinases and are of particular importance in late-stage nervous system development, where they are involved in cell positioning and morphology modulation (segmentation, axon guidance, cell migration, angiogenesis, limb development and cancer). Ephrin receptors have been implicated in antidepressant treatment response (Ising *et al.* 2009) and has been shown to regulate neural plasticity in the hippocampus (Yamaguchi and Pasquale, 2004).

Lastly, the following were networks downregulated by citalopram in the striatum:

1. Carbonic anhydrase II, VDAC 1, ATP5A, DHPR, MPTP complex.
2. DJ-1, PKM2, PGAM1, TPI1, IDH3B (Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial).

3. G3P2, DJ-1, p53, androgen receptor, c-Myc.

Downregulation of pathway 1 signals an alteration in mitochondrial enzymes induced by citalopram which is different to the one observed in the brain of haloperidol treated animals. The expression of carbonic anhydrase II was reduced in the brain of citalopram and haloperidol treated animals, whereas voltage-dependent anion selective protein 1 and ATP5A were decreased in the citalopram group but not in the haloperidol one. This difference may explain the somewhat opposing effects antipsychotics and antidepressants have on neuronal plasticity, synaptogenesis and architecture.

Downregulation of pathway 2 may indicate an alteration in energy (glucose) metabolism since phosphoglycerate mutase 1 catalyzes step 8 of glycolisis (3-phosphoglycerate to 2-phosphoglycerate); TPI1 is an enzyme on the glycolisis/gluconeogenesis pathway that catalizes the isomerisation of glyceraldehides 3-phosphate (G3P) and dihydroxy-acetone phosphate (DHAP); and IDH3B is an enzyme of the Krebs cycle that catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate. Lower baseline cerebral metabolism and greater decrease following citalopram treatment has recently been reported as predictor of good response to chronic treatment in the elderly (Smith *et al.* 2011).

In pathway 3, downregulation of glyceraldehyde-3-phosphate dehydrogenase (G3P2); DJ-1 (a neuroprotective transcriptional co-activator); p53 (a tumour suppressor protein); the androgen receptor and c-Myc (a transcription factor) suggests a role in regulation of cell cycle, gene expression and induction (prevention if downregulated) of apoptosis, which may explain the neurogenesis observed with SSRI treatment.

Thus, in plasma haloperidol decreased the expression of fibrinogen, piruvate kinase M1/M2, angiotensinogen and C3 while citalopram increased it; and increased plasminogen while citalopram did not alter its expression. Whereas in striatum, haloperidol increased aspartate amino transferase, isocitrate dehydrogenase and ATP synthase subunit α, mitochondrial while citalopram decreased their expression. Haloperidol also decreased the expression of succinate dehydrogenase and of dihydropyrimidinase-related protein 2, both of which were not altered in the striatum of citalopram treated animals. citalopram increased the expression of carbonic anhydrase 2 and 1 in plasma, an effect not observed with haloperidol; and decreased the expression of piruvate kinase M1/M2 and voltage dependent anion selective channel proteins 1 and 2 in striatum despite the lack of effect of haloperidol in those.

Haloperidol and citalopram appear to affect protein and ion binding, insulin signalling and glucose metabolism, response to hypoxia and apoptosis.

Haloperidol's main effect in plasma was seen on dopaminergic and cholinergic transmission, cell proliferation, migration and apoptosis, whereas in striatum, it appeared to modify mitochondrial function, glucose metabolism, cell cycle and apoptosis.

In plasma, citalopram altered hemostasis, lipid metabolism, mitochondrial function, modulation of HPA axis, apoptosis and calcium-signalling, whereas in striatum, it affected mitochondrial function, glucose metabolism, cell cycle and gene expression, maybe conferring an anti-apoptotic effect.

Human homologs of most of the proteins identified in this study had been reported as being altered in SCZ proteomic studies. For instance, the levels of apolipoprotein A-IV were significantly decreased in the CSF of schizophrenic patients compared to controls and the levels of haptoglobin, fibrinogen, complement C3 were also altered in the disease group according to one study (Jiang *et al.* 2003). Equally, dihydropyrimidinase-related protein 2 (detected in the striatum analysis), had been reported as being decreased in the brains of SCZ and bipolar disorder patients (Johnston-Wilson *et al.* 2000), triosephosphate isomerase has been reported as decreased in bipolar disorder (Behan *et al.* 2009) and is altered by clozapine treatment (Baig *et al.* 2010), phosphoglycerate mutase 1, has been found to be decreased in postmortem prefrontal cortex of SCZ patients (Novikova *et al.* 2006) and ubiquitin carboxyl-terminal hydrolase isozyme L1 was found to be decreased in SCZ (Prabakaran *et al.* 2004) and in bipolar disorder (Behan *et al.* 2009). These differences, which were considered attributable to the pathophysiology of the disorder, could be better explained as being the effect of medication.

As a result, caution should be taken when interpreting proteomic results from studies involving patients on medication. In addition, these results illustrate the importance of using animal and cellular models in SCZ research.

6.2.3. Finding biomarkers for psychosis in plasma

In this chapter, plasma samples from 30 cases presenting for the first time with psychosis to the South London and Maudsley NHS Foundation Trust's psychiatric services were compared to 30 well-matched controls from the local catchment-area population using 2DPAGE and LC/MS/MS. Proteins found to be differentially expressed between cases and controls were S100A8, afamin, peroxiredoxin 1, clusterin and gelsolin. Validation was performed using immunoassay detection methods on gelsolin and S100B in an independent sample from the same population. Gelsolin levels were found to be decreased in cases, while S100B appeared to be upregulated.

The gelsolin gene is located in 9q33, in a locus reported to be implicated in modifying the clinical course and severity of SCZ (Fanous *et al.* 2007). Defects in this gene also cause familial amyloidosis Finnish type (FAF), whereby systemic deposition of amyloid by the secreted (not cytoplasmic) form of the protein is found in most tissues resulting in a characteristic triad of ophthalmological, neurologic and dermatological symptoms (Fanous *et al.* 2007). DISC1, a well-established and studied schizophrenia risk gene as discussed previously, has been recently shown to act downstream of the amyloid precursor protein to regulate cortical precursor cell migration (Young-Pearse *et al.* 2010) and a reduction in its function induces perturbed mitochondrial calcium dynamics (Park *et al.* 2010).

A recent study from the Lovestone group has shown that AD patients also displayed significantly lower plasma gelsolin levels compared to non-denmented controls (NDC) subjects and correlated with disease progression rate estimated by Mini-Mental Status Examination (Guntert *et al.* 2010). Interestingly, gelsolin (a calcium-regulated protein that exerts its effect in the assembly and disassembly of actin filaments) expression, has been shown to be increased in prefrontal cortices of people affected with Down syndrome (Ji *et al.* 2009), which are known to have an increased risk of AD.

S100 proteins are Ca^{2+} -binding proteins which are located in chromosomes 1q21, 21q22, Xp22, 4p16 and 5q14 and are present exclusively in vertebrates (Sedaghat and Notopoulos, 2008). These proteins localize in cytoplasm and/or nucleus of a wide range of cells and are involved in cellular processes such as contraction, motility, cell growth and differentiation, cell cycle progression, transcription, structural organisation of membranes, dynamics of cytoskeleton constituents, protection from oxidative cell damage, protein phosphorylation and secretion (Santamaria-Kisiel *et al.* 2006). S100A8 is a calcium-binding protein whose gene is located in 1q21, an area in which microdeletions have been linked to SCZ in a recent study (Li *et al.* 2011). This specific protein may inhibit the enzyme casein kinase, which regulates signal transduction pathways including the phosphorylation of Dishevelled (Dsh) in the Wnt signalling pathway (Davidson *et al.* 2005) and acts in a molecular pathway that regulates circadian rhythm (Eide *et al.* 2005). Abnormalities of Wnt signalling in SCZ have already been suggested (Cotter *et al.* 1998) and circadian rhythm alterations in these patients have been well documented since the 1960s (for a recent review, see (Lamont *et al.* 2010)). S100A8 regulates cellular chemotaxis and has been implicated in cystic fibrosis, rheumatoid arthritis, inflammation and asthma. Asthma has been claimed to be underrepresented in people with SCZ compared to patients suffering from affective disorders (Nasr *et al.* 1981); and there is a long-observed inverse correlation between rheumatoid arthritis and SCZ (Eaton *et al.* 1992; Mellsop *et al.* 1974; Mohamed *et al.* 1982).

S100A8 levels were initially measured using a commercial antibody but no differences were found between cases and controls. This negative result was attributed to the poor quality of the antibody and a measurement of another one of these 25 proteins, S100B, in plasma was conducted using an ELISA kit from MERCK. S100B is a recognized biomarker for traumatic brain injury, several chronic neurological diseases (schizophrenia, bipolar disorder, Alzheimer's disease) plus may have utility as a biomarker in other disease states, such as cerebral palsy, Down syndrome, multiple sclerosis and metastatic melanoma. S100B is the most abundant member of the S100 family expressed in the brain, predominantly synthesized and secreted by astrocytes. In addition, S100B, maps to 22q21, an area of linkage for Schizophrenia and BPAD with psychosis (Liu *et al.* 2005; Roche *et al.* 2007) which has been identified as regulatory for presynaptic calcium signalling and synaptic plasticity in a mouse model of 22q11 deletion syndrome (Earls *et al.* 2010). Increased extracellular levels of S100B in the brain are indicative of neuronal injury or underlying chronic inflammation that can be subsequently measured in the peripheral circulation, after passing through the blood brain barrier. S100B, has previously been associated with SCZ in CSF (Rothermundt *et al.* 2004), and found to be increased in medication-free patients with a negative correlation with illness duration, indicating that a neurodegenerative process may take place in a subset of people suffering from SCZ (Lara *et al.* 2001).

Thus, classic proteomic techniques can be effectively used in SCZ research and have great promise for discovery of altered signalling pathways and molecular processes. The results of this study suggest that a subset of people presenting with psychotic might be suffering from underlying altered calcium signaling.

6.3. Thesis main findings

A consistent classical proteomic approach has been used to examine the proteome of a molecular model of the disease, the effects of medication in vivo in the plasma and striatum of F344 rats and in the plasma of SCZ patients.

1. Converging evidence implicates DHPR in SCZ. DISC1 downregulation in progenitor neural cells resulted in reduced enzyme expression. DHPR deficiency manifests itself by BH4 deficiency, leading to impaired biosynthesis of dopamine and serotonin. Haloperidol reduced the expression of this enzyme in the striatum of F344 rats, showing that the enzyme is a therapeutic target of the drug, which would act by reducing the synthesis of dopamine in an attempt to compensate for excessive dopaminergic transmission.

2. PPIA, the protein found upregulated in the DISC1 study, indicated a potential dysregulation of calcium signalling pathways which was confirmed by the validation of gelsolin and S100B levels in the plasma of SCZ patients. Gelsolin and S100B levels were altered in the plasma of a subset of patients experiencing psychotic symptoms. This peripheral signal may indicate a disease process linked to calcium signalling dysregulation that can be detected in plasma using proteomic techniques. Nevertheless, this signal may not be specific to SCZ as gelsolin levels have been shown to be reduced in the plasma of patients with AD and to correlate with disease progression (Guntert *et al.* 2010), and were increased in brain by the effect of age and also in prefrontal cortex of individuals with Down syndrome (Ji *et al.* 2009). S100B, a calcium-binding protein produced and secreted by astrocytes has also been previously associated with SCZ (Lara *et al.* 2001; Rothermundt *et al.* 2004) and with primary insulin resistance in SCZ (Steiner *et al.* 2010), but also with AD (Peskind *et al.* 2001), with multi-trauma patients without head injury (Anderson *et al.* 2001) , brain injury in children (Berger *et al.* 2002), bipolar disorder patients (Machado-Vieira *et al.* 2002), SSRI response and depression (Tramontina *et al.* 2008).

3. Antagonistic effects between haloperidol and citalopram were observed in both plasma and striatum of the treated rats. The main pathways in which haloperidol and citalopram showed opposing expression effects are glucose and lipid metabolism, mitochondrial function, immune response, coagulation, calcium signalling and apoptosis.

4. The proteins identified in the plasma of people with SCZ could be classified as belonging to one of the following groups: protease inhibitors, lipid transport, inflammation, cytoskeletal, cell cycle, neurotrophic factors, glucose metabolism and apoptosis. Several of the proteins identified in this thesis in the plasma of patients with SCZ are consistent with previous reports although they do appear to be common to other neuropsychiatric disorders. For instance, clusterin which was identified in the plasma of people with SCZ had its gene recently reported as being linked to SCZ (Zhou *et al.* 2010), has also been associated with the severity, pathology and progression of AD (Thambisetty *et al.* 2010a). Afamin, α-1-microglobulin, APOE, transthyretin, α-1-antitrypsin were upregulated while clusterin was downregulated in the plasma of pregnant mothers carrying Down syndrome foetuses (Kolialexi *et al.* 2008).

Taken together, these findings suggest that schizophrenia could be better understood as a heterogeneous group of disorders in which abnormal neurotransmitter synthesis, glucose and lipid metabolism, mitochondrial dysfunction, immune system disregulation, pro-apoptotic events and intracellular cell signalling systems result in a deficient synaptic transmission. It is known that synaptic transmission is dependent on a number of factors such as (1) the Bell-

Magendie law (which asserts that synaptic transmission occurs in one direction) that relay on membrane integrity and adequate transmembrane ion-exchange for the action potential to travel along the axon; (2) synaptic fatigue, by which a synapse will cease functioning if its transmitter release is quicker than its synthesis (a process that may occur if NT synthesis is impeded by the downregulation of certain crucial enzymes); (3) Ca^{2+} and Mg^{2+} concentrations, by which a reduction in the former or an increment in the latter will reduce the amplitude of the synaptic potential, thus affecting NT release in the synapse; and (4) alterations in pH, by which alkalosis (pH increment) increases neurotransmision and acidosis (pH reduction) decreases it. Acidosis is frequently seen in dysregulation of glucose metabolism, such as in untreated diabetes.

This dysfunction at the level of the synapse is ultimately responsible for a deficient neuronal transmission, leading to abhorrent sensorial input, which eventually leads to the emergence of psychopathology.

Schizophrenia is a useful concept when applied to convey clinically relevant information about the quality and severity of psychopathology, prognosis and recommended treatment algorithms for the symptomatic relief of behavioural manifestations of a series of unknown brain disorders. The phenotypes that clinicians identify with this diagnostic category are likely to be phenocopies resulting from complex interactions between diverse, albeit probably convergent molecular pathways and environmental influxes.

At the core of this abnormality there may be an undetermined number of genes, spread throughout the genome and coding for proteins with structural or functional roles, which additive effect disrupt brain development largely by altering the delicate interplay existing at the core of molecular and signalling events. This in turn may lead to aberrant cytostructure and cellular physiology, rendering neuronal transmission largely inefficient. In order to investigate these signalling pathways and establish their effects on the aberrant synaptic transmission, it is crucial to determine downstream effects from the gene to the synapse.

Table 37. Proteins identified in more than one of the studies in this thesis.

Chapter 3 DISC1	Chapter 5 F344 plasma	Chapter 5 F344 striatum	Chapter 6 Plasma Human
Dihydropteridine reductase	ATP synthase subunit beta, mitochondrial	ATP synthase subunit alpha, mitochondrial	Ig kappa chain C region
	Carbonic anhydrase 2	Carbonic anhydrase 2	Ig kappa chain V-II region TEW
	Ig kappa chain C region, A allele	Dihydropteridine reductase	Ig lambda chain C regions
	Ig lambda-2 chain C region	Pyruvate kinase isozymes M1/M2	
	Pyruvate kinase isozymes M1/M2		
	Tubulin alpha-1C chain		

6.4. Limitations

There are several limitations to this study. Some of them are inherent to the nature of the analyte (protein), the sample under study from which those were extracted (cell line, brain tissue and plasma of F344 animals), and human plasma; others are limitations imposed by the techniques used for the analysis (2DPAGE, Progenesis Samespots, LC/MS/MS); and lastly there are limitations to the inferences that can be drawn from comparing data emerging from the different studies in this thesis.

1. **Proteins:** Proteins are complex analytes to study. They have different isoforms, are secreted and metabolised in different ways, they may form complexes when binding to other proteins or other substrates such as lipids, and they have active and inactive forms and a potentially large number of post-translational modifications. In addition, degradation of the sample inevitably occurs through the process of sampling, processing, storing and analysing. Proteins are subjected to anticoagulants, preservers, protease inhibitors, extreme cold temperatures and thawing processes and denaturation prior to being analysed. This experimental error that inevitably occurs in laboratory experiments can be minimised by ensuring that the sampling, processing, storing and analysis of the sample is equal for cases and the controls. This was attempted by always running combined (i.e. cases and controls at the same time) batches in all experiments.

2. **Cell lines:** Human cortical neural progenitor cells (CTXOE03) are not neurons. Moreover, they are not 'schizophrenic' neurons despite having had their DISC-1 gene knock down by RNAi. It is clear that most cases of SCZ are not due to DISC-1 haploinsufficiency and it could be argued that the changes observed do not give an accurate picture of what the protein expression is like in the brain of people with SCZ. However, the human neural stem cell line, CTXOE03, is derived from human somatic stem cells following genetic modification with a conditional immortalizing gene, c-mycERTAM. This transgene generates a fusion protein that stimulates cell proliferation in the presence of a synthetic drug 4-hydroxy-tamoxifen (4-OHT). The cell line is clonal, expands rapidly in culture (doubling time 50-60 h) and has a normal human karyotype (46 XY). In the absence of growth factors and 4-OHT, the cells undergo growth arrest and differentiate into neurons and astrocytes (Pollock et al. 2006). The information obtained form this experiment is limited to ascertaining molecular pathways that may be altered as a result of DISC-1 dysfunction. Nevertheless, if said pathways were found to be affected by antipsychotic medication in the case of the animal study, or to differ from cases and controls in the case of the human plasma study, there will be a strong argument to advocate for their involvement in the aetiology of a subset of psychotic disorders.

3. **Animals:** The Fischer F344 inbred rat is a standard laboratory animal strain that has been used in toxicology, immunology, ophthalmology, oncology, aging, and neurology and psychiatry research extensively. The animals were housed in a purpose-built animal house and were bred, treated and sacrificed according to current practices by skilled researchers. Following the implantation of the mini-pumps, the animals had to be relocated to prevent control-treated animals attacking the ones that were sedated by medication. By doing this, cages went from housing one specimen of each treatment group (as would ideally have been the case) to housing specimens from the same experimental arm. Although the general conditions of the animal house ensured that each cage was exposed to the same conditions, inevitably each case had its own individual set of circumstances that were not shared between the groups. In addition, rodents will establish a hierarchy between them when housed together. Dominance in rodents has been linked to variation in serotonin levels (Kostowski *et al.* 1984) and to influence neurogenesis in the dentate gyrus of Sprague Dawley rats (Kozorovitskiy and Gould, 2004). However unlikely these are to have affected the overall outcome of the experiment (it was also an inevitable circumstance), in future studies, more attention should be paid to these factors. Thus, the protein analysis performed in this study attempted to examine molecular changes induced by the effects of medication. There is an obvious difference between the physiology of a rodent and that of humans, but there are also well preserved common molecular pathways that are homologous and examining those in an animal model is an established research approach. The information obtained, though had to be carefully contextualised and thought of as guidance for the human study, not as a validation of their results.

4. **Plasma:** Plasma is that it has a large dynamic range, exceeding 10^{10} (from albumin at around 45 mg/mL to some cytokine at 1-10 pg/mL), whereas proteomic techniques have a narrower level of detection 10^4 to 10^6 and candidate biomarkers are likely to be low abundant. In addition, alterations in plasma protein expression are inevitable during the experimental design. Plasma samples were kept on ice and carefully processed within a few hours of venipuncture. They were thawed on ice and never subjected to more than 3 freeze-thaw cycles. Nonetheless, when aliquots were taken from the principal EDTA tube, several smaller Eppendorf tubes were filled with plasma and immediately frozen. These Eppendorf tubes were labelled 1,2,…,and 7. The first tubes contained plasma that was in the top section of the tube and the last ones contained plasma that was in close contact with the Buffy coat and the RBC fraction. It is possible that different protein fractions are present at different levels in plasma the primary tube. And thus, analysis of plasma sample should have taken this into account.

5. **2DPAGE:** This technique was selected over DIGE as our lab did not have the capabilities or the expertise to use it. SILAC couldn't be used for human studies because it is performed in cell cultured media and it was felt that it was important to keep the same experimental design throughout. The limitations of this technique are that it is labour intensive, time consuming, and mostly not automated, requiring a high level of laboratory skills. A relatively large amount of sample (>300 µg of total protein) is needed to be able to excised it from the gel and analysed by mass spectrometry. It has limited reproducibility, and a smaller dynamic range than some other separation methods. However, the strength of 2-D electrophoresis has been also recognized: improved robustness and unique ability to analyze complete native proteins with all modifications. Better results with 2DPAGE will be obtained with samples with a limited range of protein expression (i.e. an organelle, subcellular fractions, protein complex, or cerebrospinal fluid). 2DPAGE is good for looking at proteins within the mass range of 20 - 250 kDa and pI of 3 - 11. All the experiments in this thesis were run using the same equipment, the same protocol and were all performed by me. Each run had a mixture of cases and controls, so that experimental error could be minimised. However, differences in the length of the second run were noticeable between the warmer months and during in winter as the room temperature in our lab was not controlled.

6. **Progenesis SameSpots:** *SameSpots* still estimates spot boundaries, eliminates missing data, and reduces technical variability in the spot quantifications. The spot detection algorithm utilized by the *SameSpots* interface in the Progenesis package appears, at times, to have difficulty splitting overlapping spots in dense regions of the gel.

7. **Spot picking:** Manual spot picking involved certain amount of subjective identification by two experienced researchers. The template used for the identification was always an analytical gel, whereas the spots were picked from a preparatory gel, which was overloaded to ensure ulterior protein identification. The use of preparatory gels was needed as glutaraldehyde, which is incompatible with LC/MS/MS, was used to improve focusing on the analytical gels. This was necessary to optimise image analysis. It is reasonable to consider that certain keratin contamination from human skin and hair occurred.

8. **Mass spectrometry:** All experiments were performed at the core facility by an experienced researcher (Steve Lynham) using LC/MS/MS as better identification and better sequence coverage is obtained than by using MALDI-MS. The general limitations of this technique are centred on the following, interacting aspects of clinical LC-MS/MS: highly manual workflows, complexity of operation and maintenance of instrumentation, sample

throughput limits, insufficient detection sensitivity for some analytes and problems with detection specificity. Nonetheless, standard operational procedures have been optimised at the core facility and these caveats had been previously addressed.

9. **Interpreting data:** Inferring information across studies performed in different samples (i.e. cell culture, animal model and human samples) is possible but by no means straight forward. Caution should be used when making inferences from the results of those experiments as the changes observed in protein expression may not be found 'in vivo'. It is possible to imagine a situation by which proteins more easily detectable because of their quantity and the scope of detection of current techniques, are also the ones subject the most to modifications and degradations though the experimental design.

Analysing the protein expression of a knock down genetic model in a cell line, the effects of antipsychotic treatment in an animal model and the plasma of people with schizophrenia with 2DPAGE showed that the technique works best with 'simpler' samples such as cell culture or brain. In comparison to these, plasma is a complex and difficult tissue to analyse.

6.5. Future directions

We are closer to finding the molecular alterations underlying psychotic symptomatology as well as clarifying the mechanisms of antipsychotic action. The task nonetheless, is a complex and multifaceted one in which combined approaches using different techniques should be used in order to fully understand the intricacies of the disease pathway leading from genetic predisposition to the behavioural manifestation of psychotic symptoms. In order to do so, a linear step-wise approach such as the one detailed below could be used (Figure 45).

1. Proteomic analysis of genetic models of the disease. Schizophrenia has a strong genetic component (Gottesman and Shields, 1967; Sullivan *et al.* 2003) and GWAS studies are being conducted under the underlying assumption of 'common disease, common variant' hypothesis in an attempt to find the disease genes. Most variants identified so far confer relatively small increments in risk and explain only a small proportion of familial clustering (Manolio *et al.* 2009). Candidate genes prioritised according to GWAS significance could be downregulated in neuronal progenitor and glial cell lines using RNAi and their protein expression examined using proteomic techniques. The author and co-authors of the DISC1 study have received funding to conduct these experiments for ZNF804A and TCF4 in CTXOE03 cells.

2. Examining the effects of current antipsychotic treatment in vitro. In vitro models for schizophrenia are currently being developed using stem cells (Brennand *et al.* 2011), astrocytes (Martins-De-Souza *et al.* 2011; Quincozes-Santos *et al.* 2010) and neuronal cultured cell lines (Ono *et al.* 2010). The effects of medication or psychosis inducing agents such as cannabis or amphetamines could be further examined using proteomic techniques.

3. Drosophila models could be employed to study the effects of risk genes (van and van, 2011) (Figure 44) and have been routinely used in human neurodegeneration research (Hirth, 2010). This approach has been successfully applied to assert the regulatory effect of dysbindin over synaptic homeostasis (Dickman and Davis, 2009). DISC1 has already been successfully model in flies and up to 3 disease genes could be examined for epigenetic interactions simultaneously (Furukubo-Tokunaga, 2009). In addition, behavioural, anatomical and physiological studies can be performed in this system.

4. In vivo mammalian studies. If the candidate gene is shown to (1) alter cellular morphology/physiology of neuronal or glial cell lines; (2) alter fly brain morphology for which a homolog alteration in patients with schizophrenia has been shown; (3) alter fly behavioural patterns; (4) modify synaptic transmission or (5) alters signalling pathways

previously identified in schizophrenia, an animal mammalian model such as inbred rats or mice, would be helpful in establishing morphological, physiological and behavioural alterations. Proteomic techniques could be used to examine the brain (including specific isolation and analysis of the synaptosome), blood and other tissues of genetically altered animals. Animal models have already been succesfully used to clarify complex mechanisms (Baker, 2011) and to study the interplay between biological, behavioural and environmental influences in schizophrenia and autism (Nagai *et al.* 2011; Tordjman *et al.* 2007). In addition, tissue from antipsychotic treated mice has already been used to discriminate protein changes resulting from the disease process from those being induced by medication (Focking *et al.* 2011). Dysbindin-1 mutant mice have shown deficits in auditory-evoked response adaptation and prepulse inhibition of startle similar to patterns seen in SCZ (Carlson *et al.* 2011). This suggests a potential underlying mechanism that could be further explored using proteomic approaches. This approach has recently shown that reduced p35 expression in mice synapses results in protein changes similar to SCZ post mortem-brain (Engmann *et al.* 2011).

5. Signalling pathways will be identified, followed downstream and annotated using pathway analysis (English *et al.* 2011). A targeted proteomics approach could then be used used to identify targets in human brain tissue. A 2-PAGE proteomic reference map for the prefrontal cortex region of the human brain has recently been published (McManus *et al.* 2010) and contain most of the analytes identified in this thesis in the animal study, suggesting that inferences from animal models to human brain can be made.

6. Lastly, potential plasma candidates could be explored using high-throughput targeted proteomics in human plasma. This guided proteomic approach has already been successfully employed in SCZ. Multiplex molecular profiling approaches have started to appear in plasma studies in SCZ research (Schwarz *et al.* 2011a) and more recently, multiplex immunoanalyses carried out in the serum of SCZ and BPAD patients suggested that there are distinct alterations in the prodromal stages of the disorder (Schwarz *et al.* 2011b).

Proteomic research in neuropsychiatric disorders in general and in psychotic disorders in particular holds great promise for the future. Since the introduction of classic proteomic techniques to study psychiatric disorders and the commencement of SWISS-2DPAGE (a database holding data on proteins identified on 2DPAGE maps) in 1993 (Appel *et al.* 1993), these techniques have shown alterations at the protein level in the plasma, serum, CSF and brain of patients with SCZ and psychotic disorders. Most of the proteins identified are involved

in cytoskeletal functions, energy metabolism, cell signalling, and synaptic transmission, suggesting that a basic cell dysregulation operates at the core of the etiological disease process leading to psychotic disorders.

Proteomic techniques will continue being applied to investigating disease associated pathways through modelling the disorder in vitro and in vivo models and with the use of automated throughput targeted approaches in human samples. This newly acquired knowledge could eventually lead to a re-classification of mental illness that could leap across, not only to other neuropsychiatric disorders such as Alzheimer's or Parkinson's disease but possibly even encompass systemic disorders such as diabetes mellitus or rheumatoid arthritis, which may share very similar molecular alterations in a different target organ/tissue, resulting in the different disease phenotypes observed.

In addition, proteomic techniques will also play a major role in clarifying the mechanisms of action of medication as well as their side-effect profiles, contributing to stratified medicine by improving the management of groups of patients with shared molecular baseline characteristics. These patients would be screened using molecular markers and available therapeutic options could be selected on the basis of their predicted response and tolerability, ending the trial and error process that clinicians and their patients currently have to endure in the hope of finding a medication that, at best, will alleviate some of the distressing symptoms the patients continue to suffer from.

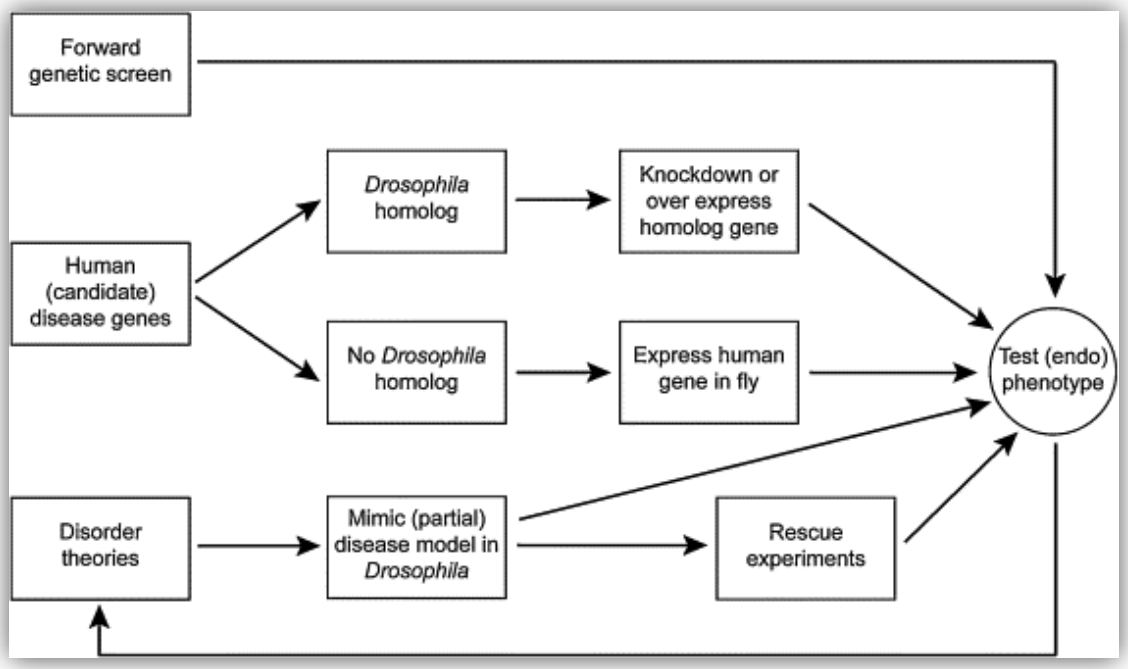

Figure 33. Strategies for using Drosophila in the study of disease.
Entry points are genetic screen, candidate genes and modelling theories about the aetiology or ontogeny of the disorder in the fly.

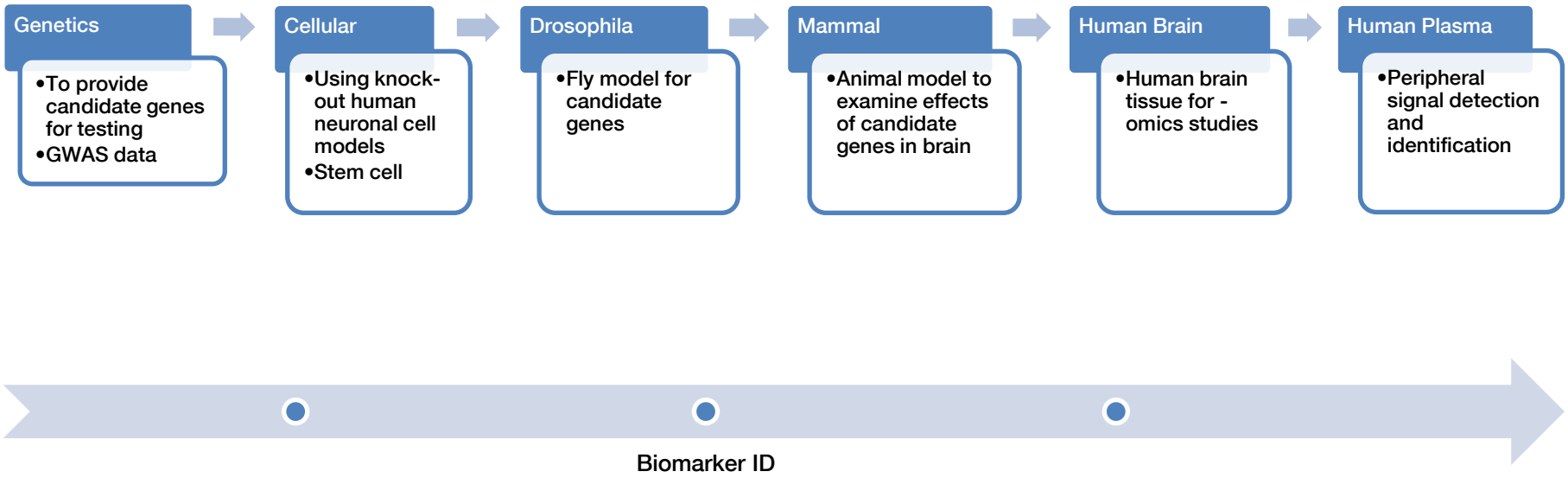

Figure 34. Workflow of research plan.

REFERENCES

Abram, S. R., Kruskal, J. B., Allen, G. S., Burns, R. S., Parker, R. & Tulipan, N. (1990). Alterations in prealbumin concentration after adrenal autotransplantation for Parkinson's disease. ***Exp. Neurol.*** **108, 130-135.**

Aghajanian, G. K. & Marek, G. J. (1997). Serotonin induces excitatory postsynaptic potentials in apical dendrites of neocortical pyramidal cells. ***Neuropharmacology*** **36, 589-599.**

Alderman, C. P., Seshadri, P. & Ben-Tovim, D. I. (1996). Effects of serotonin reuptake inhibitors on hemostasis. ***Ann. Pharmacother.*** **30, 1232-1234.**

Allen, J., Davey, H. M., Broadhurst, D., Heald, J. K., Rowland, J. J., Oliver, S. G. & Kell, D. B. (2003). High-throughput classification of yeast mutants for functional genomics using metabolic footprinting. ***Nat. Biotechnol.*** **21, 692-696.**

Anderson, N. L. & Anderson, N. G. (2002). The human plasma proteome: history, character, and diagnostic prospects. ***Mol Cell Proteomics.*** **1, 845-867.**

Anderson, R. E., Hansson, L. O., Nilsson, O., jlai-Merzoug, R. & Settergren, G. (2001). High serum S100B levels for trauma patients without head injuries. ***Neurosurgery*** **48, 1255-1258.**

Andrade, C., Sandarsh, S., Chethan, K. B. & Nagesh, K. S. (2010). Serotonin reuptake inhibitor antidepressants and abnormal bleeding: a review for clinicians and a reconsideration of mechanisms. ***J Clin. Psychiatry*** **71, 1565-1575.**

Appel, R. D., Sanchez, J. C., Bairoch, A., Golaz, O., Miu, M., Vargas, J. R. & Hochstrasser, D. F. (1993). SWISS-2DPAGE: a database of two-dimensional gel electrophoresis images. ***Electrophoresis*** **14, 1232-1238.**

Arnold, S. E., Franz, B. R., Trojanowski, J. Q., Moberg, P. J. & Gur, R. E. (1996). Glial fibrillary acidic protein-immunoreactive astrocytosis in elderly patients with schizophrenia and dementia. ***Acta Neuropathol.*** **91, 269-277.**

Asarnow, J. R. (1988). Children at risk for schizophrenia: converging lines of evidence. ***Schizophr. Bull.*** **14, 613-631.**

Baig, M. R., Navaira, E., Escamilla, M. A., Raventos, H. & Walss-Bass, C. (2010). Clozapine treatment causes oxidation of proteins involved in energy metabolism in lymphoblastoid cells: a possible mechanism for antipsychotic-induced metabolic alterations. ***J Psychiatr. Pract.*** **16, 325-333.**

Bajestan, S. N., Sabouri, A. H., Nakamura, M., Takashima, H., Keikhaee, M. R., Behdani, F., Fayyazi, M. R., Sargolzaee, M. R., Bajestan, M. N., Sabouri, Z., Khayami, E., Haghighi, S., Hashemi, S. B., Eiraku, N., Tufani, H., Najmabadi, H., Arimura, K., Sano, A. & Osame, M. (2006). Association of AKT1 haplotype with the risk of schizophrenia in Iranian population. ***Am J Med. Genet. B Neuropsychiatr. Genet.*** **141B, 383-386.**

Baker, M. (2011). Animal models: inside the minds of mice and men. ***Nature*** **475, 123-128.**

Baki, L., Shioi, J., Wen, P., Shao, Z., Schwarzman, A., Gama-Sosa, M., Neve, R. & Robakis, N. K. (2004). PS1 activates PI3K thus inhibiting GSK-3 activity and tau overphosphorylation: effects of FAD mutations. ***EMBO J*** **23, 2586-2596.**

Banki, C. M., Bissette, G., Arato, M. & Nemeroff, C. B. (1988). Elevation of immunoreactive CSF TRH in depressed patients. *Am J Psychiatry* 145, 1526-1531.

Banki, C. M., Karmacsi, L., Bissette, G. & Nemeroff, C. B. (1992). CSF corticotropin releasing hormone, somatostatin, and thyrotropin releasing hormone in schizophrenia. *Psychiatry Res.* 43, 13-21.

Barbosa, I. G., Huguet, R. B., Neves, F. S., Reis, H. J., Bauer, M. E., Janka, Z., Palotas, A. & Teixeira, A. L. (2010). Impaired nerve growth factor homeostasis in patients with bipolar disorder. *World J Biol. Psychiatry* .

Barratt, J. & Topham, P. (2007). Urine proteomics: the present and future of measuring urinary protein components in disease. *CMAJ.* 177, 361-368.

Bayer, T. A., Buslei, R., Havas, L. & Falkai, P. (1999). Evidence for activation of microglia in patients with psychiatric illnesses. *Neurosci. Lett.* 271, 126-128.

Behan, A. T., Byrne, C., Dunn, M. J., Cagney, G. & Cotter, D. R. (2009). Proteomic analysis of membrane microdomain-associated proteins in the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder reveals alterations in LAMP, STXBP1 and BASP1 protein expression. *Mol Psychiatry* 14, 601-613.

Beinfeld, M. C. & Garver, D. L. (1991). Concentration of cholecystokinin in cerebrospinal fluid is decreased in psychosis: relationship to symptoms and drug response. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 15, 601-609.

Beloosesky, Y., Salman, H., Bergman, M., Bessler, H. & Djaldetti, M. (2002). Cytokine levels and phagocytic activity in patients with Alzheimer's disease. *Gerontology* 48, 128-132.

Ben-Shachar, D. (2009). The interplay between mitochondrial complex I, dopamine and Sp1 in schizophrenia. *J Neural Transm.* 116, 1383-1396.

Benitez-King, G., Ortiz-Lopez, L., Jimenez-Rubio, G. & Ramirez-Rodriguez, G. (2010). Haloperidol causes cytoskeletal collapse in N1E-115 cells through tau hyperphosphorylation induced by oxidative stress: Implications for neurodevelopment. *Eur. J Pharmacol.* 644, 24-31.

Berger, R. P., Pierce, M. C., Wisniewski, S. R., Adelson, P. D., Clark, R. S., Ruppel, R. A. & Kochanek, P. M. (2002). Neuron-specific enolase and S100B in cerebrospinal fluid after severe traumatic brain injury in infants and children. *Pediatrics* 109, E31.

Bessler, H., Djaldetti, R., Salman, H., Bergman, M. & Djaldetti, M. (1999). IL-1 beta, IL-2, IL-6 and TNF-alpha production by peripheral blood mononuclear cells from patients with Parkinson's disease. *Biomed. Pharmacother.* 53, 141-145.

Binder, E. B., Kinkead, B., Owens, M. J. & Nemeroff, C. B. (2001). The role of neurotensin in the pathophysiology of schizophrenia and the mechanism of action of antipsychotic drugs. *Biol. Psychiatry* 50, 856-872.

Biomarkers Definition Working Group (2001). Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 69.

Blackwood, D. H., Fordyce, A., Walker, M. T., St, C. D., Porteous, D. J. & Muir, W. J. (2001). Schizophrenia and affective disorders--cosegregation with a translocation at

chromosome 1q42 that directly disrupts brain-expressed genes: clinical and P300 findings in a family. ***Am J Hum. Genet.*** **69, 428-433.**

Blackwood, D. H. & Muir, W. J. (2004). Clinical phenotypes associated with DISC1, a candidate gene for schizophrenia. ***Neurotox. Res.*** **6, 35-41.**

Blennow, K. (2004). Cerebrospinal fluid protein biomarkers for Alzheimer's disease. ***NeuroRx.*** **1, 213-225.**

Blennow, K., Bogdanovic, N., Alafuzoff, I., Ekman, R. & Davidsson, P. (1996). Synaptic pathology in Alzheimer's disease: relation to severity of dementia, but not to senile plaques, neurofibrillary tangles, or the ApoE4 allele. ***J Neural Transm.*** **103, 603-618.**

Blennow, K., Bogdanovic, N., Gottfries, C. G. & Davidsson, P. (1999). The growth-associated protein GAP-43 is increased in the hippocampus and in the gyrus cinguli in schizophrenia. ***J Mol Neurosci.*** **13, 101-109.**

Blennow, K., Davidsson, P., Wallin, A. & Ekman, R. (1995). Chromogranin A in cerebrospinal fluid: a biochemical marker for synaptic degeneration in Alzheimer's disease? ***Dementia*** **6, 306-311.**

Bourdillon, R. E., Clarke, C. A., Ridges, A. P., Sheppard, P. M., Harper, P. & Leslie, S. A. (1965). 'Pink spot' in the urine of schizophrenics. ***Nature*** **208, 453-455.**

Braaten, D. & Luban, J. (2001). Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. ***EMBO J*** **20, 1300-1309.**

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. ***Anal. Biochem.*** **72, 248-254.**

Brandon, N. J., Handford, E. J., Schurov, I., Rain, J. C., Pelling, M., Duran-Jimeniz, B., Camargo, L. M., Oliver, K. R., Beher, D., Shearman, M. S. & Whiting, P. J. (2004). Disrupted in Schizophrenia 1 and Nudel form a neurodevelopmentally regulated protein complex: implications for schizophrenia and other major neurological disorders. ***Mol Cell Neurosci.*** **25, 42-55.**

Brandon, N. J., Millar, J. K., Korth, C., Sive, H., Singh, K. K. & Sawa, A. (2009). Understanding the role of DISC1 in psychiatric disease and during normal development. ***J Neurosci.*** **29, 12768-12775.**

Brennand, K. J., Simone, A., Jou, J., Gelboin-Burkhart, C., Tran, N., Sangar, S., Li, Y., Mu, Y., Chen, G., Yu, D., McCarthy, S., Sebat, J. & Gage, F. H. (2011). Modelling schizophrenia using human induced pluripotent stem cells. ***Nature*** **473, 221-225.**

Broadbelt, K., Ramprasaud, A. & Jones, L. B. (2006). Evidence of altered neurogranin immunoreactivity in areas 9 and 32 of schizophrenic prefrontal cortex. ***Schizophr. Res.*** **87, 6-14.**

Brodacki, B., Staszewski, J., Toczylowska, B., Kozlowska, E., Drela, N., Chalimoniuk, M. & Stepien, A. (2008). Serum interleukin (IL-2, IL-10, IL-6, IL-4), TNFalpha, and INFgamma concentrations are elevated in patients with atypical and idiopathic parkinsonism. ***Neurosci. Lett.*** **441, 158-162.**

Brown, A. S. & Derkits, E. J. (2010). Prenatal infection and schizophrenia: a review of epidemiologic and translational studies. *Am J Psychiatry* 167, 261-280.

Brown, R., Colter, N., Corsellis, J. A., Crow, T. J., Frith, C. D., Jagoe, R., Johnstone, E. C. & Marsh, L. (1986). Postmortem evidence of structural brain changes in schizophrenia. Differences in brain weight, temporal horn area, and parahippocampal gyrus compared with affective disorder. *Arch. Gen. Psychiatry* 43, 36-42.

Buchanan, R. W., Javitt, D. C., Marder, S. R., Schooler, N. R., Gold, J. M., McMahon, R. P., Heresco-Levy, U. & Carpenter, W. T. (2007). The Cognitive and Negative Symptoms in Schizophrenia Trial (CONSIST): the efficacy of glutamatergic agents for negative symptoms and cognitive impairments. *Am J Psychiatry* 164, 1593-1602.

Buckland, P. R., O'Donovan, M. C. & McGuffin, P. (1992). Changes in dopa decarboxylase mRNA but not tyrosine hydroxylase mRNA levels in rat brain following antipsychotic treatment. *Psychopharmacology (Berl)* 108, 98-102.

Byne, W., Buchsbaum, M. S., Mattiace, L. A., Hazlett, E. A., Kemether, E., Elhakem, S. L., Purohit, D. P., Haroutunian, V. & Jones, L. (2002). Postmortem assessment of thalamic nuclear volumes in subjects with schizophrenia. *Am J Psychiatry* 159, 59-65.

Calarco, J. A., Xing, Y., Caceres, M., Calarco, J. P., Xiao, X., Pan, Q., Lee, C., Preuss, T. M. & Blencowe, B. J. (2007). Global analysis of alternative splicing differences between humans and chimpanzees. *Genes Dev.* 21, 2963-2975.

Callahan, L. M., Selski, D. J., Martzen, M. R., Cheetham, J. E. & Coleman, P. D. (1994). Preliminary evidence: decreased GAP-43 message in tangle-bearing neurons relative to adjacent tangle-free neurons in Alzheimer's disease parahippocampal gyrus. *Neurobiol. Aging* 15, 381-386.

Camargo, L. M., Collura, V., Rain, J. C., Mizuguchi, K., Hermjakob, H., Kerrien, S., Bonnert, T. P., Whiting, P. J. & Brandon, N. J. (2007). Disrupted in Schizophrenia 1 Interactome: evidence for the close connectivity of risk genes and a potential synaptic basis for schizophrenia. *Mol Psychiatry* 12, 74-86.

Cande, C., Vahsen, N., Kouranti, I., Schmitt, E., Daugas, E., Spahr, C., Luban, J., Kroemer, R. T., Giordanetto, F., Garrido, C., Penninger, J. M. & Kroemer, G. (2004). AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. *Oncogene* 23, 1514-1521.

Cannon, T. D., Kaprio, J., Lonnqvist, J., Huttunen, M. & Koskenvuo, M. (1998). The genetic epidemiology of schizophrenia in a Finnish twin cohort. A population-based modeling study. *Arch. Gen. Psychiatry* 55, 67-74.

Cantor-Graae, E. (2007). The contribution of social factors to the development of schizophrenia: a review of recent findings. *Can. J Psychiatry* 52, 277-286.

Carlson, G. C., Talbot, K., Halene, T. B., Gandal, M. J., Kazi, H. A., Schlosser, L., Phung, Q. H., Gur, R. E., Arnold, S. E. & Siegel, S. J. (2011). Dysbindin-1 mutant mice implicate reduced fast-phasic inhibition as a final common disease mechanism in schizophrenia. *Proc. Natl. Acad. Sci. U. S. A* .

Carlsson, A., Kehr, W. & Lindqvist, M. (1977). Agonist--antagonist interaction on dopamine receptors in brain, as reflected in the rates of tyrosine and tryptophan hydroxylation. ***J Neural Transm.*** **40, 99-113.**

Cech, N. B. & Enke, C. G. (2001). Practical implications of some recent studies in electrospray ionization fundamentals. ***Mass Spectrom. Rev.*** **20, 362-387.**

Chagnon, Y. C., Roy, M. A., Bureau, A., Merette, C. & Maziade, M. (2008). Differential RNA expression between schizophrenic patients and controls of the dystrobrevin binding protein 1 and neuregulin 1 genes in immortalized lymphocytes. ***Schizophr. Res.*** **100, 281-290.**

Chang, L., Friedman, J., Ernst, T., Zhong, K., Tsopelas, N. D. & Davis, K. (2007). Brain metabolite abnormalities in the white matter of elderly schizophrenic subjects: implication for glial dysfunction. ***Biol. Psychiatry*** **62, 1396-1404.**

Chen, H. M., Lin, C. Y. & Wang, V. (2011). Amyloid P component as a plasma marker for Parkinson's disease identified by a proteomic approach. ***Clin. Biochem.*** **44, 377-385.**

Chertkow, Y., Weinreb, O., Youdim, M. B. & Silver, H. (2007). Gene expression changes in peripheral mononuclear cells from schizophrenic patients treated with a combination of antipsychotic with fluvoxamine. ***Prog. Neuropsychopharmacol. Biol. Psychiatry*** **31, 1356-1362.**

Chubb, J. E., Bradshaw, N. J., Soares, D. C., Porteous, D. J. & Millar, J. K. (2008). The DISC locus in psychiatric illness. ***Mol Psychiatry*** **13, 36-64.**

Cichon, S., Craddock, N., Daly, M., Faraone, S. V., Gejman, P. V., Kelsoe, J., Lehner, T., Levinson, D. F., Moran, A., Sklar, P. & Sullivan, P. F. (2009). Genomewide association studies: history, rationale, and prospects for psychiatric disorders. ***Am. J. Psychiatry*** **166, 540-556.**

Cocco, C., D'Amato, F., Noli, B., Ledda, A., Brancia, C., Bongioanni, P. & Ferri, G. L. (2010). Distribution of VGF peptides in the human cortex and their selective changes in Parkinson's and Alzheimer's diseases. ***J Anat.*** **217, 683-693.**

Copeland, L. A., Parchman, M. L., Zeber, J. E., Lawrence, V. A., Downs, J. R. & Miller, A. L. (2010). Prediabetes assessment and follow-up in older veterans with schizophrenia. ***Am J Geriatr. Psychiatry*** **18, 887-896.**

Cosi, C., Waget, A., Rollet, K., Tesori, V. & Newman-Tancredi, A. (2005). Clozapine, ziprasidone and aripiprazole but not haloperidol protect against kainic acid-induced lesion of the striatum in mice, in vivo: role of 5-HT1A receptor activation. ***Brain Res.*** **1043, 32-41.**

Cotter, D., Kerwin, R., al-Sarraji, S., Brion, J. P., Chadwich, A., Lovestone, S., Anderton, B. & Everall, I. (1998). Abnormalities of Wnt signalling in schizophrenia--evidence for neurodevelopmental abnormality. ***Neuroreport*** **9, 1379-1383.**

Craddock, N., Kendler, K., Neale, M., Nurnberger, J., Purcell, S., Rietschel, M., Perlis, R., Santangelo, S. L., Schulze, T., Smoller, J. W. & Thapar, A. (2009). Dissecting the phenotype in genome-wide association studies of psychiatric illness. ***Br. J. Psychiatry*** **195, 97-99.**

Craddock, N., O'Donovan, M. C. & Owen, M. J. (2006). Genes for schizophrenia and bipolar disorder? Implications for psychiatric nosology. *Schizophr Bull* 32.

Crepel, A., Breckpot, J., Fryns, J. P., De la, M. W., Steyaert, J., Devriendt, K. & Peeters, H. (2010). DISC1 duplication in two brothers with autism and mild mental retardation. *Clin. Genet.* 77, 389-394.

Crow, T. J., Ball, J., Bloom, S. R., Brown, R., Bruton, C. J., Colter, N., Frith, C. D., Johnstone, E. C., Owens, D. G. & Roberts, G. W. (1989). Schizophrenia as an anomaly of development of cerebral asymmetry. A postmortem study and a proposal concerning the genetic basis of the disease. *Arch. Gen. Psychiatry* 46, 1145-1150.

Cubells, J. F., Sun, X., Li, W., Bonsall, R. W., McGrath, J. A., Avramopoulos, D., Lasseter, V. K., Wolyniec, P. S., Tang, Y. L., Mercer, K., Pulver, A. E. & Elston, R. C. (2011). Linkage analysis of plasma dopamine beta-hydroxylase activity in families of patients with schizophrenia. *Hum. Genet.*

Cunha, A. B., Frey, B. N., Andreazza, A. C., Goi, J. D., Rosa, A. R., Goncalves, C. A., Santin, A. & Kapczinski, F. (2006). Serum brain-derived neurotrophic factor is decreased in bipolar disorder during depressive and manic episodes. *Neurosci. Lett.* 398, 215-219.

Cutler, N. R., Kay, A. D., Marangos, P. J. & Burg, C. (1986). Cerebrospinal fluid neuron-specific enolase is reduced in Alzheimer's disease. *Arch. Neurol.* 43, 153-154.

Dalal, M. A., Schuld, A. & Pollmacher, T. (2003). Lower CSF orexin A (hypocretin-1) levels in patients with schizophrenia treated with haloperidol compared to unmedicated subjects. *Mol Psychiatry* 8, 836-837.

Dammann, O. & Leviton, A. (1997). Maternal intrauterine infection, cytokines, and brain damage in the preterm newborn. *Pediatr. Res.* 42, 1-8.

Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., Glinka, A. & Niehrs, C. (2005). Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* 438, 867-872.

Davidson, M. R., Larsen, J. E., Yang, I. A., Hayward, N. K., Clarke, B. E., Duhig, E. E., Passmore, L. H., Bowman, R. V. & Fong, K. M. (2010). MicroRNA-218 is deleted and downregulated in lung squamous cell carcinoma. *PLoS. One.* 5, e12560.

Davidsson, P. & Blennow, K. (1998). Neurochemical dissection of synaptic pathology in Alzheimer's disease. *Int. Psychogeriatr.* 10, 11-23.

Davidsson, P., Bogdanovic, N., Lannfelt, L. & Blennow, K. (2001). Reduced expression of amyloid precursor protein, presenilin-1 and rab3a in cortical brain regions in Alzheimer's disease. *Dement. Geriatr. Cogn Disord.* 12, 243-250.

Davidsson, P., Puchades, M. & Blennow, K. (1999). Identification of synaptic vesicle, pre- and postsynaptic proteins in human cerebrospinal fluid using liquid-phase isoelectric focusing. *Electrophoresis* 20, 431-437.

Davis, K. L., Kahn, R. S., Ko, G. & Davidson, M. (1991). Dopamine in schizophrenia: a review and reconceptualization. *Am J Psychiatry* 148, 1474-1486.

Davis, K. L., Stewart, D. G., Friedman, J. I., Buchsbaum, M., Harvey, P. D., Hof, P. R., Buxbaum, J. & Haroutunian, V. (2003). White matter changes in schizophrenia: evidence for myelin-related dysfunction. ***Arch. Gen. Psychiatry*** **60, 443-456.**

Dean, B., Boer, S., Gibbons, A., Money, T. & Scarr, E. (2009). Recent advances in postmortem pathology and neurochemistry in schizophrenia. ***Curr. Opin. Psychiatry*** **22, 154-160.**

Dean, B., Digney, A., Sundram, S., Thomas, E. & Scarr, E. (2008). Plasma apolipoprotein E is decreased in schizophrenia spectrum and bipolar disorder. ***Psychiatry Res.*** **158, 75-78.**

DeLisi, L. E., Shaw, S. H., Crow, T. J., Shields, G., Smith, A. B., Larach, V. W., Wellman, N., Loftus, J., Nanthakumar, B., Razi, K., Stewart, J., Comazzi, M., Vita, A., Heffner, T. & Sherrington, R. (2002). A genome-wide scan for linkage to chromosomal regions in 382 sibling pairs with schizophrenia or schizoaffective disorder. ***Am J Psychiatry*** **159, 803-812.**

Dessi, F., Colle, M. A., Hauw, J. J. & Duyckaerts, C. (1997). Accumulation of SNAP-25 immunoreactive material in axons of Alzheimer's disease. ***Neuroreport*** **8, 3685-3689.**

Dickinson, D. & Harvey, P. D. (2009). Systemic hypotheses for generalized cognitive deficits in schizophrenia: a new take on an old problem. ***Schizophr. Bull.*** **35, 403-414.**

Dickman, D. K. & Davis, G. W. (2009). The schizophrenia susceptibility gene dysbindin controls synaptic homeostasis. ***Science*** **326, 1127-1130.**

Done, D. J., Crow, T. J., Johnstone, E. C. & Sacker, A. (1994). Childhood antecedents of schizophrenia and affective illness: social adjustment at ages 7 and 11. ***BMJ*** **309, 699-703.**

Doyle, A. E., Biederman, J., Ferreira, M. A., Wong, P., Smoller, J. W. & Faraone, S. V. (2010). Suggestive linkage of the child behavior checklist juvenile bipolar disorder phenotype to 1p21, 6p21, and 8q21. ***J Am Acad. Child Adolesc. Psychiatry*** **49, 378-387.**

Drouot, X., Moutereau, S., Nguyen, J. P., Lefaucheur, J. P., Creange, A., Remy, P., Goldenberg, F. & d'Ortho, M. P. (2003). Low levels of ventricular CSF orexin/hypocretin in advanced PD. ***Neurology*** **61, 540-543.**

Duan, X., Chang, J. H., Ge, S., Faulkner, R. L., Kim, J. Y., Kitabatake, Y., Liu, X. B., Yang, C. H., Jordan, J. D., Ma, D. K., Liu, C. Y., Ganesan, S., Cheng, H. J., Ming, G. L., Lu, B. & Song, H. (2007). Disrupted-In-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. ***Cell*** **130, 1146-1158.**

Earls, L. R., Bayazitov, I. T., Fricke, R. G., Berry, R. B., Illingworth, E., Mittleman, G. & Zakharenko, S. S. (2010). Dysregulation of presynaptic calcium and synaptic plasticity in a mouse model of 22q11 deletion syndrome. ***J Neurosci.*** **30, 15843-15855.**

Eastwood, S. L. & Harrison, P. J. (1998). Hippocampal and cortical growth-associated protein-43 messenger RNA in schizophrenia. ***Neuroscience*** **86, 437-448.**

Eastwood, S. L. & Harrison, P. J. (2001). Synaptic pathology in the anterior cingulate cortex in schizophrenia and mood disorders. A review and a Western blot study of synaptophysin, GAP-43 and the complexins. ***Brain Res. Bull.*** **55, 569-578.**

Eaton, W. W., Hayward, C. & Ram, R. (1992). Schizophrenia and rheumatoid arthritis: a review. *Schizophr. Res.* 6, 181-192.

Eide, E. J., Woolf, M. F., Kang, H., Woolf, P., Hurst, W., Camacho, F., Vielhaber, E. L., Giovanni, A. & Virshup, D. M. (2005). Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation. *Mol Cell Biol.* 25, 2795-2807.

Ekelund, J., Hovatta, I., Parker, A., Paunio, T., Varilo, T., Martin, R., Suhonen, J., Ellonen, P., Chan, G., Sinsheimer, J. S., Sobel, E., Juvonen, H., Arajarvi, R., Partonen, T., Suvisaari, J., Lonnqvist, J., Meyer, J. & Peltonen, L. (2001). Chromosome 1 loci in Finnish schizophrenia families. *Hum. Mol Genet.* 10, 1611-1617.

Elovaara, I., Maury, C. P. & Palo, J. (1986). Serum amyloid A protein, albumin and prealbumin in Alzheimer's disease and in demented patients with Down's syndrome. *Acta Neurol. Scand.* 74, 245-250.

Emberley, E. D., Niu, Y., Curtis, L., Troup, S., Mandal, S. K., Myers, J. N., Gibson, S. B., Murphy, L. C. & Watson, P. H. (2005). The S100A7-c-Jun activation domain binding protein 1 pathway enhances prosurvival pathways in breast cancer. *Cancer Res.* 65, 5696-5702.

English, J. A., Pennington, K., Dunn, M. J. & Cotter, D. R. (2011). The neuroproteomics of schizophrenia. *Biol. Psychiatry* 69, 163-172.

Engmann, O., Hortobagyi, T., Pidsley, R., Troakes, C., Bernstein, H. G., Kreutz, M. R., Mill, J., Nikolic, M. & Giese, K. P. (2011). Schizophrenia is associated with dysregulation of a Cdk5 activator that regulates synaptic protein expression and cognition. *Brain* 134, 2408-2421.

Fanous, A. H. & Kendler, K. S. (2008). Genetics of clinical features and subtypes of schizophrenia: a review of the recent literature. *Curr. Psychiatry Rep.* 10, 164-170.

Fanous, A. H., Neale, M. C., Webb, B. T., Straub, R. E., Amdur, R. L., O'Neill, F. A., Walsh, D., Riley, B. P. & Kendler, K. S. (2007). A genome-wide scan for modifier loci in schizophrenia. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 144B, 589-595.

Farrah, T., Deutsch, E. W., Omenn, G. S., Campbell, D. S., Sun, Z., Bletz, J. A., Mallick, P., Katz, J. E., Malmstrom, J., Ossola, R., Watts, J. D., Lin, B., Zhang, H., Moritz, R. L. & Aebersold, R. (2011). A high-confidence human plasma proteome reference set with estimated concentrations in PeptideAtlas. *Mol Cell Proteomics.* 10, M110.

Fatemi, S. H., Kroll, J. L. & Stary, J. M. (2001). Altered levels of Reelin and its isoforms in schizophrenia and mood disorders. *Neuroreport* 12, 3209-3215.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. & Whitehouse, C. M. (1989). Electrospray ionization for mass spectrometry of large biomolecules. *Science* 246, 64-71.

Ferrer, I., Gomez-Isla, T., Puig, B., Freixes, M., Ribe, E., Dalfo, E. & Avila, J. (2005). Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies. *Curr. Alzheimer Res.* 2, 3-18.

Flechtner-Mors, M., Jenkinson, C. P., Alt, A., Adler, G. & Ditschuneit, H. H. (2008). Metabolism in adipose tissue in response to citalopram and trimipramine treatment--an in situ microdialysis study. *J Psychiatr. Res.* 42, 578-586.

Focking, M., Dicker, P., English, J. A., Schubert, K. O., Dunn, M. J. & Cotter, D. R. (2011). Common proteomic changes in the hippocampus in schizophrenia and bipolar disorder and particular evidence for involvement of cornu ammonis regions 2 and 3. *Arch. Gen. Psychiatry* 68, 477-488.

Fonteh, A. N., Harrington, R. J., Huhmer, A. F., Biringer, R. G., Riggins, J. N. & Harrington, M. G. (2006). Identification of disease markers in human cerebrospinal fluid using lipidomic and proteomic methods. *Dis Markers* 22.

Fritze, J., Koronakis, P. & Riederer, P. (1988). Erythrocyte membrane proteins in psychiatric disorders and controls. *J Affect. Disord.* 15, 187-190.

Fukuda, S., Hashimoto, R., Ohi, K., Yamaguti, K., Nakatomi, Y., Yasuda, Y., Kamino, K., Takeda, M., Tajima, S., Kuratsune, H., Nishizawa, Y. & Watanabe, Y. (2010). A functional polymorphism in the disrupted-in schizophrenia 1 gene is associated with chronic fatigue syndrome. *Life Sci.* 86, 722-725.

Fukunaka, Y., Shinkai, T., Hwang, R., Hori, H., Utsunomiya, K., Sakata, S., Naoe, Y., Shimizu, K., Matsumoto, C., Ohmori, O. & Nakamura, J. (2007). The orexin 1 receptor (HCRTR1) gene as a susceptibility gene contributing to polydipsia-hyponatremia in schizophrenia. *Neuromolecular. Med.* 9, 292-297.

Furukubo-Tokunaga, K. (2009). Modeling schizophrenia in flies. *Prog. Brain Res.* 179, 107-115.

Ganguli, R. & Rabin, B. S. (1989). Increased serum interleukin 2 receptor concentration in schizophrenic and brain-damaged subjects. *Arch. Gen. Psychiatry* 46, 292.

Garver, D. L., Beinfeld, M. C. & Yao, J. K. (1990). Cholecystokinin, dopamine and schizophrenia. *Psychopharmacol. Bull.* 26, 377-380.

Garver, D. L., Tamas, R. L. & Holcomb, J. A. (2003). Elevated interleukin-6 in the cerebrospinal fluid of a previously delineated schizophrenia subtype. *Neuropsychopharmacology* 28, 1515-1520.

Geddes, J. R. & Lawrie, S. M. (1995). Obstetric complications and schizophrenia: a meta-analysis. *Br. J Psychiatry* 167, 786-793.

Giovacchini, G., Squitieri, F., Esmaeilzadeh, M., Milano, A., Mansi, L. & Ciarmiello, A. (2011). PET translates neurophysiology into images: A review to stimulate a network between neuroimaging and basic research. *J Cell Physiol* 226, 948-961.

Gitler, A. D., Bevis, B. J., Shorter, J., Strathearn, K. E., Hamamichi, S., Su, L. J., Caldwell, K. A., Caldwell, G. A., Rochet, J. C., McCaffery, J. M., Barlowe, C. & Lindquist, S. (2008). The Parkinson's disease protein alpha-synuclein disrupts cellular Rab homeostasis. *Proc. Natl. Acad. Sci. U. S. A* 105, 145-150.

Gladkevich, A., Kauffman, H. F. & Korf, J. (2004). Lymphocytes as a neural probe: potential for studying psychiatric disorders. *Prog Neuropsychopharmacol Biol Psychiatry* 28.

Glantz, L. A., Gilmore, J. H., Lieberman, J. A. & Jarskog, L. F. (2006). Apoptotic mechanisms and the synaptic pathology of schizophrenia. *Schizophr. Res.* 81, 47-63.

Glantz, L. A. & Lewis, D. A. (1997). Reduction of synaptophysin immunoreactivity in the prefrontal cortex of subjects with schizophrenia. Regional and diagnostic specificity. *Arch. Gen. Psychiatry* 54, 943-952.

Glantz, L. A. & Lewis, D. A. (2000). Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Arch. Gen. Psychiatry* 57, 65-73.

Glavan, G., Schliebs, R. & Zivin, M. (2009). Synaptotagmins in neurodegeneration. *Anat. Rec. (Hoboken.)* 292, 1849-1862.

Goodacre, R. (2005). Metabolomics shows the way to new discoveries. *Genome Biol.* 6, 354.

Gottesman, I. I. & Shields, J. (1967). A polygenic theory of schizophrenia. *Proc. Natl. Acad. Sci. U. S. A* 58, 199-205.

Gray, L. J., Dean, B., Kronsbein, H. C., Robinson, P. J. & Scarr, E. (2010). Region and diagnosis-specific changes in synaptic proteins in schizophrenia and bipolar I disorder. *Psychiatry Res.* 178, 374-380.

Greber, S., Lubec, G., Cairns, N. & Fountoulakis, M. (1999). Decreased levels of synaptosomal associated protein 25 in the brain of patients with Down syndrome and Alzheimer's disease. *Electrophoresis* 20, 928-934.

Green, M. F., Satz, P., Gaier, D. J., Ganzell, S. & Kharabi, F. (1989). Minor physical anomalies in schizophrenia. *Schizophr. Bull.* 15, 91-99.

Grossman, M., Farmer, J., Leight, S., Work, M., Moore, P., Van, D., V, Pratico, D., Clark, C. M., Coslett, H. B., Chatterjee, A., Gee, J., Trojanowski, J. Q. & Lee, V. M. (2005). Cerebrospinal fluid profile in frontotemporal dementia and Alzheimer's disease. *Ann. Neurol.* 57, 721-729.

Guest, P. C., Wang, L., Harris, L. W., Burling, K., Levin, Y., Ernst, A., Wayland, M. T., Umrania, Y., Herberth, M., Koethe, D., van Beveren, J. M., Rothermundt, M., McAllister, G., Leweke, F. M., Steiner, J. & Bahn, S. (2010). Increased levels of circulating insulin-related peptides in first-onset, antipsychotic naive schizophrenia patients. *Mol Psychiatry* 15, 118-119.

Guntert, A., Campbell, J., Saleem, M., O'Brien, D. P., Thompson, A. J., Byers, H. L., Ward, M. A. & Lovestone, S. (2010). Plasma gelsolin is decreased and correlates with rate of decline in Alzheimer's disease. *J Alzheimers. Dis.* 21, 585-596.

Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H. & Aebersold, R. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 17, 994-999.

Halberstadt, A. L. (1995). The phencyclidine-glutamate model of schizophrenia. *Clin. Neuropharmacol.* 18, 237-249.

Hamshere, M. L., Bennett, P., Williams, N., Segurado, R., Cardno, A., Norton, N., Lambert, D., Williams, H., Kirov, G., Corvin, A., Holmans, P., Jones, L., Jones, I., Gill, M., O'Donovan, M. C., Owen, M. J. & Craddock, N. (2005). Genomewide linkage scan in schizoaffective disorder: significant evidence for linkage at 1q42 close to DISC1, and suggestive evidence at 22q11 and 19p13. *Arch. Gen. Psychiatry* 62, 1081-1088.

Hamshere, M. L., Schulze, T. G., Schumacher, J., Corvin, A., Owen, M. J., Jamra, R. A., Propping, P., Maier, W., Diaz, G., Mayoral, F., Rivas, F., Jones, I., Jones, L., Kirov, G., Gill, M., Holmans, P. A., Nothen, M. M., Cichon, S., Rietschel, M. & Craddock, N. (2009). Mood-incongruent psychosis in bipolar disorder: conditional linkage analysis shows genome-wide suggestive linkage at 1q32.3, 7p13 and 20q13.31. *Bipolar. Disord.* 11, 610-620.

Hanger, D. P., Hughes, K., Woodgett, J. R., Brion, J. P. & Anderton, B. H. (1992). Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localisation of the kinase. *Neurosci. Lett.* 147, 58-62.

Harrison, P. J. (1999). The neuropathology of schizophrenia. A critical review of the data and their interpretation. *Brain* 122 (Pt 4), 593-624.

Harrison, P. J. & Eastwood, S. L. (2001). Neuropathological studies of synaptic connectivity in the hippocampal formation in schizophrenia. *Hippocampus* 11, 508-519.

Hashimoto, R., Numakawa, T., Ohnishi, T., Kumamaru, E., Yagasaki, Y., Ishimoto, T., Mori, T., Nemoto, K., Adachi, N., Izumi, A., Chiba, S., Noguchi, H., Suzuki, T., Iwata, N., Ozaki, N., Taguchi, T., Kamiya, A., Kosuga, A., Tatsumi, M., Kamijima, K., Weinberger, D. R., Sawa, A. & Kunugi, H. (2006). Impact of the DISC1 Ser704Cys polymorphism on risk for major depression, brain morphology and ERK signaling. *Hum. Mol Genet.* 15, 3024-3033.

Hattori, K., Fukuzako, H., Hashiguchi, T., Hamada, S., Murata, Y., Isosaka, T., Yuasa, S. & Yagi, T. (2009). Decreased expression of Fyn protein and disbalanced alternative splicing patterns in platelets from patients with schizophrenia. *Psychiatry Res.* 168, 119-128.

Heckers, S. (2001). Neuroimaging studies of the hippocampus in schizophrenia. *Hippocampus* 11, 520-528.

Henley, S. M., Bates, G. P. & Tabrizi, S. J. (2005). Biomarkers for neurodegenerative diseases. *Curr. Opin. Neurol.* 18, 698-705.

Hennah, W., Varilo, T., Kestila, M., Paunio, T., Arajarvi, R., Haukka, J., Parker, A., Martin, R., Levitzky, S., Partonen, T., Meyer, J., Lonnqvist, J., Peltonen, L. & Ekelund, J. (2003). Haplotype transmission analysis provides evidence of association for DISC1 to schizophrenia and suggests sex-dependent effects. *Hum. Mol Genet.* 12, 3151-3159.

Heresco-Levy, U. (2003). Glutamatergic neurotransmission modulation and the mechanisms of antipsychotic atypicality. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 1113-1123.

Hesketh, S. A., Leggett, J. D. & Jessop, D. S. (2007). Chronic citalopram treatment does not sensitize the adrenal gland to ACTH (1-24) in rats. *J Psychopharmacol.* 21, 885-887.

Heston, L. L. (1966). Psychiatric disorders in foster home reared children of schizophrenic mothers. *Br. J Psychiatry* 112, 819-825.

Hikida, T., Jaaro-Peled, H., Seshadri, S., Oishi, K., Hookway, C., Kong, S., Wu, D., Xue, R., Andrade, M., Tankou, S., Mori, S., Gallagher, M., Ishizuka, K., Pletnikov, M., Kida, S. & Sawa, A. (2007). Dominant-negative DISC1 transgenic mice display schizophrenia-associated phenotypes detected by measures translatable to humans. *Proc. Natl. Acad. Sci. U. S. A* 104, 14501-14506.

Hirth, F. (2010). Drosophila melanogaster in the study of human neurodegeneration. *CNS. Neurol. Disord. Drug Targets.* 9, 504-523.

Ho, L., Sharma, N., Blackman, L., Festa, E., Reddy, G. & Pasinetti, G. M. (2005). From proteomics to biomarker discovery in Alzheimer's disease. *Brain Res. Brain Res. Rev.* 48, 360-369.

Hochstrasser, D. F., Patchornik, A. & Merril, C. R. (1988). Development of polyacrylamide gels that improve the separation of proteins and their detection by silver staining. *Anal. Biochem.* 173, 412-423.

Hock, C., Heese, K., Muller-Spahn, F., Huber, P., Riesen, W., Nitsch, R. M. & Otten, U. (2000a). Increased CSF levels of nerve growth factor in patients with Alzheimer's disease. *Neurology* 54, 2009-2011.

Hock, C. H., Heese, K., Olivieri, G., Hulette, C. H., Rosenberg, C., Nitsch, R. M. & Otten, U. (2000b). Alterations in neurotrophins and neurotrophin receptors in Alzheimer's disease. *J Neural Transm. Suppl* 59, 171-174.

Hodgkinson, C. A., Goldman, D., Jaeger, J., Persaud, S., Kane, J. M., Lipsky, R. H. & Malhotra, A. K. (2004). Disrupted in schizophrenia 1 (DISC1): association with schizophrenia, schizoaffective disorder, and bipolar disorder. *Am J Hum. Genet.* 75, 862-872.

Holmes, E., Tsang, T. M., Huang, J. T., Leweke, F. M., Koethe, D., Gerth, C. W., Nolden, B. M., Gross, S., Schreiber, D., Nicholson, J. K. & Bahn, S. (2006). Metabolic profiling of CSF: evidence that early intervention may impact on disease progression and outcome in schizophrenia. *PLoS Med* 3.

Honer, W. G., Falkai, P., Chen, C., Arango, V., Mann, J. J. & Dwork, A. J. (1999). Synaptic and plasticity-associated proteins in anterior frontal cortex in severe mental illness. *Neuroscience* 91, 1247-1255.

Hong, M., Chen, D. C., Klein, P. S. & Lee, V. M. (1997). Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3. *J Biol. Chem.* 272, 25326-25332.

Honkakoski, P., Zelko, I., Sueyoshi, T. & Negishi, M. (1998). The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol.* 18, 5652-5658.

Horlings, H. M., Lai, C., Nuyten, D. S., Halfwerk, H., Kristel, P., van, B. E., Joosse, S. A., Klijn, C., Nederlof, P. M., Reinders, M. J., Wessels, L. F. & van, d., V (2010). Integration of DNA copy number alterations and prognostic gene expression signatures in breast cancer patients. *Clin. Cancer Res.* 16, 651-663.

Hortin, G. L. (2006). The MALDI-TOF mass spectrometric view of the plasma proteome and peptidome. *Clin. Chem.* 52, 1223-1237.

Hovatta, I., Varilo, T., Suvisaari, J., Terwilliger, J. D., Ollikainen, V., Arajarvi, R., Juvonen, H., Kokko-Sahin, M. L., Vaisanen, L., Mannila, H., Lonnqvist, J. & Peltonen, L. (1999). A genomewide screen for schizophrenia genes in an isolated Finnish subpopulation, suggesting multiple susceptibility loci. *Am J Hum. Genet.* 65, 1114-1124.

Hu, Y. Q., Liebl, D. J., Dluzen, D. E. & Koo, P. H. (1998). Inhibition of dopamine and choline acetyltransferase concentrations in rat CNS neurons by rat alpha 1- and alpha 2-macroglobulins. *J Neurosci. Res.* 51, 541-550.

Huang, J. T., Leweke, F. M., Oxley, D., Wang, L., Harris, N., Koethe, D., Gerth, C. W., Nolden, B. M., Gross, S., Schreiber, D., Reed, B. & Bahn, S. (2006a). Disease biomarkers in cerebrospinal fluid of patients with first-onset psychosis. ***PLoS. Med.*** **3, e428.**

Huang, J. T., Leweke, F. M., Tsang, T. M., Koethe, D., Kranaster, L., Gerth, C. W., Gross, S., Schreiber, D., Ruhrmann, S., Schultze-Lutter, F., Klosterkotter, J., Holmes, E. & Bahn, S. (2007). CSF metabolic and proteomic profiles in patients prodromal for psychosis. ***PLoS ONE*** **2.**

Huang, J. T., Wang, L., Prabakaran, S., Wengenroth, M., Lockstone, H. E., Koethe, D., Gerth, C. W., Gross, S., Schreiber, D., Lilley, K., Wayland, M., Oxley, D., Leweke, F. M. & Bahn, S. (2008). Independent protein-profiling studies show a decrease in apolipoprotein A1 levels in schizophrenia CSF, brain and peripheral tissues. ***Mol Psychiatry*** **13.**

Huang, W. J., Liu, Z. C., Wei, W., Wang, G. H., Wu, J. G. & Zhu, F. (2006b). Human endogenous retroviral pol RNA and protein detected and identified in the blood of individuals with schizophrenia. ***Schizophr. Res.*** **83, 193-199.**

Huberman, M., Shalit, F., Roth-Deri, I., Gutman, B., Brodie, C., Kott, E. & Sredni, B. (1994). Correlation of cytokine secretion by mononuclear cells of Alzheimer patients and their disease stage. ***J Neuroimmunol.*** **52, 147-152.**

Hur, E. M. & Zhou, F. Q. (2010). GSK3 signalling in neural development. ***Nat. Rev. Neurosci.*** **11, 539-551.**

Hutchinson, T. H., Mahshid, Y., Jonsson, R., Bjorklund, C. & Kenne, K. (2008). Proteomic analysis of phospholipidosis in citalopram treated U937 cells--support for the cholesterol biosynthesis hypothesis. ***Toxicol. In Vitro*** **22, 1198-1204.**

Hye, A., Lynham, S., Thambisetty, M., Causevic, M., Campbell, J., Byers, H. L., Hooper, C., Rijsdijk, F., Tabrizi, S. J., Banner, S., Shaw, C. E., Foy, C., Poppe, M., Archer, N., Hamilton, G., Powell, J., Brown, R. G., Sham, P., Ward, M. & Lovestone, S. (2006). Proteome-based plasma biomarkers for Alzheimer's disease. ***Brain*** **129, 3042-3050.**

Ilani, T., Ben-Shachar, D., Strous, R. D., Mazor, M., Sheinkman, A., Kotler, M. & Fuchs, S. (2001). A peripheral marker for schizophrenia: Increased levels of D3 dopamine receptor mRNA in blood lymphocytes. ***Proc. Natl. Acad. Sci. U. S. A*** **98, 625-628.**

Iritani, S. (2007). Neuropathology of schizophrenia: a mini review. ***Neuropathology.*** **27, 604-608.**

Ishizuka, K., Kamiya, A., Oh, E. C., Kanki, H., Seshadri, S., Robinson, J. F., Murdoch, H., Dunlop, A. J., Kubo, K., Furukori, K., Huang, B., Zeledon, M., Hayashi-Takagi, A., Okano, H., Nakajima, K., Houslay, M. D., Katsanis, N. & Sawa, A. (2011). DISC1-dependent switch from progenitor proliferation to migration in the developing cortex. ***Nature*** **473, 92-96.**

Ising, M., Lucae, S., Binder, E. B., Bettecken, T., Uhr, M., Ripke, S., Kohli, M. A., Hennings, J. M., Horstmann, S., Kloiber, S., Menke, A., Bondy, B., Rupprecht, R., Domschke, K., Baune, B. T., Arolt, V., Rush, A. J., Holsboer, F. & Muller-Myhsok, B. (2009). A genomewide association study points to multiple loci that predict antidepressant drug treatment outcome in depression. ***Arch. Gen. Psychiatry*** **66, 966-975.**

Issa, G., Wilson, C., Terry, A. V., Jr. & Pillai, A. (2010). An inverse relationship between cortisol and BDNF levels in schizophrenia: data from human postmortem and animal studies. *Neurobiol. Dis.* 39, 327-333.

Iwai, M. & Horiuchi, M. (2009). Devil and angel in the renin-angiotensin system: ACE-angiotensin II-AT1 receptor axis vs. ACE2-angiotensin-(1-7)-Mas receptor axis. *Hypertens. Res.* 32, 533-536.

Iwazaki, T., Shibata, I., Niwa, S. & Matsumoto, I. (2004). Selective reduction of chromogranin A-like immunoreactivities in the prefrontal cortex of schizophrenic subjects: a postmortem study. *Neurosci. Lett.* 367, 293-297.

Jacobs, J. M., Adkins, J. N., Qian, W. J., Liu, T., Shen, Y., Camp, D. G. & Smith, R. D. (2005). Utilizing human blood plasma for proteomic biomarker discovery. *J Proteome. Res.* 4, 1073-1085.

James, R., Adams, R. R., Christie, S., Buchanan, S. R., Porteous, D. J. & Millar, J. K. (2004). Disrupted in Schizophrenia 1 (DISC1) is a multicompartmentalized protein that predominantly localizes to mitochondria. *Mol Cell Neurosci.* 26, 112-122.

Jensen, J. B., Jessop, D. S., Harbuz, M. S., Mork, A., Sanchez, C. & Mikkelsen, J. D. (1999). Acute and long-term treatments with the selective serotonin reuptake inhibitor citalopram modulate the HPA axis activity at different levels in male rats. *J Neuroendocrinol.* 11, 465-471.

Ji, L., Chauhan, A., Muthaiyah, B., Wegiel, J. & Chauhan, V. (2009). Gelsolin levels are increased in the brain as a function of age during normal development in children that are further increased in Down syndrome. *Alzheimer Dis. Assoc. Disord.* 23, 319-322.

Jiang, L., Lindpaintner, K., Li, H. F., Gu, N. F., Langen, H., He, L. & Fountoulakis, M. (2003). Proteomic analysis of the cerebrospinal fluid of patients with schizophrenia. *Amino. Acids* 25, 49-57.

Jin, Z. G., Lungu, A. O., Xie, L., Wang, M., Wong, C. & Berk, B. C. (2004). Cyclophilin A is a proinflammatory cytokine that activates endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 24, 1186-1191.

Jockers-Scherubl, M. C., nker-Hopfe, H., Mahlberg, R., Selig, F., Rentzsch, J., Schurer, F., Lang, U. E. & Hellweg, R. (2004). Brain-derived neurotrophic factor serum concentrations are increased in drug-naive schizophrenic patients with chronic cannabis abuse and multiple substance abuse. *Neurosci. Lett.* 371, 79-83.

Johnson, M. D., Yu, L. R., Conrads, T. P., Kinoshita, Y., Uo, T., McBee, J. K., Veenstra, T. D. & Morrison, R. S. (2005). The proteomics of neurodegeneration. *Am J Pharmacogenomics.* 5, 259-270.

Johnston-Wilson, N. L., Sims, C. D., Hofmann, J. P., Anderson, L., Shore, A. D., Torrey, E. F. & Yolken, R. H. (2000). Disease-specific alterations in frontal cortex brain proteins in schizophrenia, bipolar disorder, and major depressive disorder. The Stanley Neuropathology Consortium. *Mol Psychiatry* 5, 142-149.

Jones, P., Rodgers, B., Murray, R. & Marmot, M. (1994). Child development risk factors for adult schizophrenia in the British 1946 birth cohort. *Lancet* 344, 1398-1402.

Joo, E. J., Lee, K. Y., Jeong, S. H., Roh, M. S., Kim, S. H., Ahn, Y. M. & Kim, Y. S. (2009). AKT1 Gene Polymorphisms and Obstetric Complications in the Patients with Schizophrenia. ***Psychiatry Investig.*** **6, 102-107.**

Kaddurah-Daouk, R., McEvoy, J., Baillie, R. A., Lee, D., Yao, J. K., Doraiswamy, P. M. & Krishnan, K. R. (2007). Metabolomic mapping of atypical antipsychotic effects in schizophrenia. ***Mol Psychiatry*** **12.**

Kale, A., Joshi, S., Pillai, A., Naphade, N., Raju, M., Nasrallah, H. & Mahadik, S. P. (2009). Reduced cerebrospinal fluid and plasma nerve growth factor in drug-naive psychotic patients. ***Schizophr. Res.*** **115, 209-214.**

Kamiya, A., Kubo, K., Tomoda, T., Takaki, M., Youn, R., Ozeki, Y., Sawamura, N., Park, U., Kudo, C., Okawa, M., Ross, C. A., Hatten, M. E., Nakajima, K. & Sawa, A. (2005). A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development. ***Nat. Cell Biol.*** **7, 1167-1178.**

Kang, J. E., Lim, M. M., Bateman, R. J., Lee, J. J., Smyth, L. P., Cirrito, J. R., Fujiki, N., Nishino, S. & Holtzman, D. M. (2009). Amyloid-beta dynamics are regulated by orexin and the sleep-wake cycle. ***Science*** **326, 1005-1007.**

Kapur, S. & Mamo, D. (2003). Half a century of antipsychotics and still a central role for dopamine D2 receptors. ***Prog. Neuropsychopharmacol. Biol. Psychiatry*** **27, 1081-1090.**

Karam, C. S., Ballon, J. S., Bivens, N. M., Freyberg, Z., Girgis, R. R., Lizardi-Ortiz, J. E., Markx, S., Lieberman, J. A. & Javitch, J. A. (2010). Signaling pathways in schizophrenia: emerging targets and therapeutic strategies. ***Trends Pharmacol. Sci.*** **31, 381-390.**

Karson, C. N., Mrak, R. E., Schluterman, K. O., Sturner, W. Q., Sheng, J. G. & Griffin, W. S. (1999). Alterations in synaptic proteins and their encoding mRNAs in prefrontal cortex in schizophrenia: a possible neurochemical basis for 'hypofrontality'. ***Mol Psychiatry*** **4, 39-45.**

Keefe, R. S. (2007). Cognitive deficits in patients with schizophrenia: effects and treatment. ***J Clin. Psychiatry*** **68 Suppl 14, 8-13.**

Keefe, R. S., Bilder, R. M., Davis, S. M., Harvey, P. D., Palmer, B. W., Gold, J. M., Meltzer, H. Y., Green, M. F., Capuano, G., Stroup, T. S., McEvoy, J. P., Swartz, M. S., Rosenheck, R. A., Perkins, D. O., Davis, C. E., Hsiao, J. K. & Lieberman, J. A. (2007). Neurocognitive effects of antipsychotic medications in patients with chronic schizophrenia in the CATIE Trial. ***Arch. Gen. Psychiatry*** **64, 633-647.**

Kendler, K. S. & Diehl, S. R. (1993). The genetics of schizophrenia: a current, genetic-epidemiologic perspective. ***Schizophr. Bull.*** **19, 261-285.**

Keshavan, M. S., Tandon, R., Boutros, N. N. & Nasrallah, H. A. (2008). Schizophrenia, "just the facts": what we know in 2008 Part 3: neurobiology. ***Schizophr. Res.*** **106, 89-107.**

Kety, S. S. (1983). Mental illness in the biological and adoptive relatives of schizophrenic adoptees: findings relevant to genetic and environmental factors in etiology. ***Am J Psychiatry*** **140, 720-727.**

Kiehntopf, M., Siegmund, R. & Deufel, T. (2007). Use of SELDI-TOF mass spectrometry for identification of new biomarkers: potential and limitations. *Clin. Chem. Lab Med.* 45, 1435-1449.

Kietzmann, T., Krones-Herzig, A. & Jungermann, K. (2002). Signaling cross-talk between hypoxia and glucose via hypoxia-inducible factor 1 and glucose response elements. *Biochem. Pharmacol.* 64, 903-911.

Kilidireas, K., Latov, N., Strauss, D. H., Gorig, A. D., Hashim, G. A., Gorman, J. M. & Sadiq, S. A. (1992). Antibodies to the human 60 kDa heat-shock protein in patients with schizophrenia. *Lancet* 340, 569-572.

Kilpinen, H., Ylisaukko-Oja, T., Hennah, W., Palo, O. M., Varilo, T., Vanhala, R., Nieminen-von, W. T., von, W. L., Paunio, T. & Peltonen, L. (2008). Association of DISC1 with autism and Asperger syndrome. *Mol Psychiatry* 13, 187-196.

Kim, J. S., Kornhuber, H. H., Schmid-Burgk, W. & Holzmuller, B. (1980). Low cerebrospinal fluid glutamate in schizophrenic patients and a new hypothesis on schizophrenia. *Neurosci. Lett.* 20, 379-382.

Kim, J. S., Yoo, J. Y., Lee, K. S., Kim, H. S., Choi, J. S., Rha, H. K., Yim, S. V. & Lee, K. H. (2008). Comparative genome hybridization array analysis for sporadic Parkinson's disease. *Int. J Neurosci.* 118, 1331-1345.

Kiuru, S. (1992). Familial amyloidosis of the Finnish type (FAF). A clinical study of 30 patients. *Acta Neurol. Scand.* 86, 346-353.

Kolialexi, A., Tsangaris, G. T., Papantoniou, N., Anagnostopoulos, A. K., Vougas, K., Bagiokos, V., Antsaklis, A. & Mavrou, A. (2008). Application of proteomics for the identification of differentially expressed protein markers for Down syndrome in maternal plasma. *Prenat. Diagn.* 28, 691-698.

Kostowski, W., Plewako, M. & Bidzinski, A. (1984). Brain serotonergic neurons: their role in a form of dominance-subordination behavior in rats. *Physiol Behav.* 33, 365-371.

Kozlovsky, N., Belmaker, R. H. & Agam, G. (2000). Low GSK-3beta immunoreactivity in postmortem frontal cortex of schizophrenic patients. *Am J Psychiatry* 157, 831-833.

Kozlovsky, N., Regenold, W. T., Levine, J., Rapoport, A., Belmaker, R. H. & Agam, G. (2004). GSK-3beta in cerebrospinal fluid of schizophrenia patients. *J Neural Transm.* 111, 1093-1098.

Kozorovitskiy, Y. & Gould, E. (2004). Dominance hierarchy influences adult neurogenesis in the dentate gyrus. *J Neurosci.* 24, 6755-6759.

Kubicki, M., McCarley, R., Westin, C. F., Park, H. J., Maier, S., Kikinis, R., Jolesz, F. A. & Shenton, M. E. (2007). A review of diffusion tensor imaging studies in schizophrenia. *J Psychiatr. Res.* 41, 15-30.

Kung, L. & Roberts, R. C. (1999). Mitochondrial pathology in human schizophrenic striatum: a postmortem ultrastructural study. *Synapse* 31, 67-75.

Lamont, E. W., Coutu, D. L., Cermakian, N. & Boivin, D. B. (2010). Circadian rhythms and clock genes in psychotic disorders. *Isr. J Psychiatry Relat Sci.* 47, 27-35.

Landen, M., Grenfeldt, B., Davidsson, P., Stridsberg, M., Regland, B., Gottfries, C. G. & Blennow, K. (1999). Reduction of chromogranin A and B but not C in the cerebrospinal fluid in subjects with schizophrenia. *Eur. Neuropsychopharmacol.* 9, 311-315.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J. P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J. C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R. H., Wilson, R. K., Hillier, L. W., McPherson, J. D., Marra, M. A., Mardis, E. R., Fulton, L. A., Chinwalla, A. T., Pepin, K. H., Gish, W. R., Chissoe, S. L., Wendl, M. C., Delehaunty, K. D., Miner, T. L., Delehaunty, A., Kramer, J. B., Cook, L. L., Fulton, R. S., Johnson, D. L., Minx, P. J., Clifton, S. W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J. F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R. A., Muzny, D. M., Scherer, S. E., Bouck, J. B., Sodergren, E. J., Worley, K. C., Rives, C. M., Gorrell, J. H., Metzker, M. L., Naylor, S. L., Kucherlapati, R. S., Nelson, D. L., Weinstock, G. M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Smith, D. R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H. M., Dubois, J., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R. W., Federspiel, N. A., Abola, A. P., Proctor, M. J., Myers, R. M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D. R., Olson, M. V., Kaul, R., Raymond, C., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G. A., Athanasiou, M., Schultz, R., Roe, B. A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W. R., de la, B. M., Dedhia, N., Blocker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J. A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D. G., Burge, C. B., Cerutti, L., Chen, H. C., Church, D., Clamp, M., Copley, R. R., Doerks, T., Eddy, S. R., Eichler, E. E., Furey, T. S., Galagan, J., Gilbert, J. G., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L. S., Jones, T. A., Kasif, S., Kaspryzk, A., Kennedy, S., Kent, W. J., Kitts, P., Koonin, E. V., Korf, I., Kulp, D., Lancet, D., Lowe, T. M., McLysaght, A., Mikkelsen, T., Moran, J. V., Mulder, N., Pollara, V. J., Ponting, C. P., Schuler, G., Schultz, J., Slater, G., Smit, A. F., Stupka, E., Szustakowski, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis, J., Wheeler, R., Williams, A., Wolf, Y. I., Wolfe, K. H., Yang, S. P., Yeh, R. F., Collins, F., Guyer, M. S., Peterson, J., Felsenfeld, A., Wetterstrand, K. A., Patrinos, A., Morgan, M. J., de, J. P., Catanese, J. J., Osoegawa, K., Shizuya, H., Choi, S. & Chen, Y. J. (2001). Initial sequencing and analysis of the human genome. *Nature* 409, 860-921.

Lara, D. R., Gama, C. S., Belmonte-de-Abreu, P., Portela, L. V., Goncalves, C. A., Fonseca, M., Hauck, S. & Souza, D. O. (2001). Increased serum S100B protein in schizophrenia: a study in medication-free patients. *J Psychiatr. Res.* 35, 11-14.

Laruelle, M., bi-Dargham, A., van Dyck, C. H., Gil, R., D'Souza, C. D., Erdos, J., McCance, E., Rosenblatt, W., Fingado, C., Zoghbi, S. S., Baldwin, R. M., Seibyl, J. P., Krystal, J. H., Charney, D. S. & Innis, R. B. (1996). Single photon emission computerized

tomography imaging of amphetamine-induced dopamine release in drug-free schizophrenic subjects. *Proc. Natl. Acad. Sci. U. S. A* 93, 9235-9240.

Layton, D., Clark, D. W., Pearce, G. L. & Shakir, S. A. (2001). Is there an association between selective serotonin reuptake inhibitors and risk of abnormal bleeding? Results from a cohort study based on prescription event monitoring in England. *Eur. J Clin. Pharmacol.* 57, 167-176.

Lecourtois, M., Poirier, K., Friocourt, G., Jaglin, X., Goldenberg, A., Saugier-Veber, P., Chelly, J. & Laquerriere, A. (2010). Human lissencephaly with cerebellar hypoplasia due to mutations in TUBA1A: expansion of the foetal neuropathological phenotype. *Acta Neuropathol.* 119, 779-789.

Lee, B. H. & Kim, Y. K. (2009). Increased plasma brain-derived neurotropic factor, not nerve growth factor-Beta, in schizophrenia patients with better response to risperidone treatment. *Neuropsychobiology* 59, 51-58.

Lei, G., Xia, Y. & Johnson, K. M. (2008). The role of Akt-GSK-3beta signaling and synaptic strength in phencyclidine-induced neurodegeneration. *Neuropsychopharmacology* 33, 1343-1353.

Leite, C. M., Machado, G. J., Dornelles, R. C. & Franci, C. R. (2008). Actions of angiotensin II and dopamine in the medial preoptic area on prolactin secretion. *Physiol Res.* 57, 109-118.

Lerner, M. H. & Friedhoff, A. J. (1980). Radioimmunoassay measurement of creatine kinase BB in the serum of schizophrenic patients. *Clin. Chim. Acta* 107, 121-128.

Lesort, M., Greendorfer, A., Stockmeier, C., Johnson, G. V. & Jope, R. S. (1999). Glycogen synthase kinase-3beta, beta-catenin, and tau in postmortem bipolar brain. *J Neural Transm.* 106, 1217-1222.

Lewis, C. M., Levinson, D. F., Wise, L. H., DeLisi, L. E., Straub, R. E., Hovatta, I., Williams, N. M., Schwab, S. G., Pulver, A. E., Faraone, S. V., Brzustowicz, L. M., Kaufmann, C. A., Garver, D. L., Gurling, H. M., Lindholm, E., Coon, H., Moises, H. W., Byerley, W., Shaw, S. H., Mesen, A., Sherrington, R., O'Neill, F. A., Walsh, D., Kendler, K. S., Ekelund, J., Paunio, T., Lonnqvist, J., Peltonen, L., O'Donovan, M. C., Owen, M. J., Wildenauer, D. B., Maier, W., Nestadt, G., Blouin, J. L., Antonarakis, S. E., Mowry, B. J., Silverman, J. M., Crowe, R. R., Cloninger, C. R., Tsuang, M. T., Malaspina, D., Harkavy-Friedman, J. M., Svrakic, D. M., Bassett, A. S., Holcomb, J., Kalsi, G., McQuillin, A., Brynjolfson, J., Sigmundsson, T., Petursson, H., Jazin, E., Zoega, T. & Helgason, T. (2003). Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. *Am J Hum. Genet.* 73, 34-48.

Lewis, D. A. (2002). The human brain revisited: opportunities and challenges in postmortem studies of psychiatric disorders. *Neuropsychopharmacology* 26, 143-154.

Li, G., Peskind, E. R., Millard, S. P., Chi, P., Sokal, I., Yu, C. E., Bekris, L. M., Raskind, M. A., Galasko, D. R. & Montine, T. J. (2009). Cerebrospinal fluid concentration of brain-derived neurotrophic factor and cognitive function in non-demented subjects. *PLoS. One.* 4, e5424.

Li, J., Zhou, G., Ji, W., Feng, G., Zhao, Q., Liu, J., Li, T., Li, Y., Chen, P., Zeng, Z., Wang, T., Hu, Z., Zheng, L., Wang, Y., Shen, Y., He, L. & Shi, Y. (2011). Common Variants in the BCL9 Gene Conferring Risk of Schizophrenia. *Arch. Gen. Psychiatry* 68, 232-240.

Li, S., Wu, H., Guo, H. & Zhao, Z. (2006). Neuron-specific Enolase and myelin basic protein in cerebrospinal fluid of patients with first episode schizophrenia. ***J Huazhong. Univ Sci. Technolog. Med. Sci.*** **26, 228-230.**

Li, Z., Zhao, X., Bai, S., Wang, Z., Chen, L., Wei, Y. & Huang, C. (2008). Proteomics identification of cyclophilin a as a potential prognostic factor and therapeutic target in endometrial carcinoma. ***Mol Cell Proteomics.*** **7, 1810-1823.**

Liang, L. P. & Kaufman, S. (1998). The regulation of dopamine release from striatum slices by tetrahydrobiopterin and L-arginine-derived nitric oxide. ***Brain Res.*** **800, 181-186.**

Licinio, J., Seibyl, J. P., Altemus, M., Charney, D. S. & Krystal, J. H. (1993). Elevated CSF levels of interleukin-2 in neuroleptic-free schizophrenic patients. ***Am J Psychiatry*** **150, 1408-1410.**

Lieberman, J. A. (1999). Is schizophrenia a neurodegenerative disorder? A clinical and neurobiological perspective. ***Biol. Psychiatry*** **46, 729-739.**

Liu, J., Shi, Y., Tang, J., Guo, T., Li, X., Yang, Y., Chen, Q., Zhao, X., He, G., Feng, G., Gu, N., Zhu, S., Liu, H. & He, L. (2005). SNPs and haplotypes in the S100B gene reveal association with schizophrenia. ***Biochem. Biophys. Res. Commun.*** **328, 335-341.**

Liu, Z., Yang, F., Robotham, J. M. & Tang, H. (2009). Critical role of cyclophilin A and its prolyl-peptidyl isomerase activity in the structure and function of the hepatitis C virus replication complex. ***J Virol.*** **83, 6554-6565.**

Lorigados, P. L., Pavon, F. N., Alvarez, G. L., McRae, A., Serrano, S. T., Blanco, L. L. & Macias, G. R. (2002). Nerve growth factor levels in Parkinson disease and experimental parkinsonian rats. ***Brain Res.*** **952, 122-127.**

Lovegrove, T. D., Metcalfe, E. V., Hobbs, G. E. & Stevenson, J. A. (1965). The urinary excretion of adrenaline, noradrenaline, and 17-hydroxycorticosteroids in mental illness. ***Can. Psychiatr. Assoc. J.*** **10, 170-179.**

Lullmann-Rauch, R. & Nassberger, L. (1983). Citalopram-induced generalized lipidosis in rats. ***Acta Pharmacol. Toxicol. (Copenh)*** **52, 161-167.**

Lyons, F., Martin, M. L., Maguire, C., Jackson, A., Regan, C. M. & Shelley, R. K. (1988). The expression of an N-CAM serum fragment is positively correlated with severity of negative features in type II schizophrenia. ***Biol. Psychiatry*** **23, 769-775.**

Macgregor, S., Visscher, P. M., Knott, S. A., Thomson, P., Porteous, D. J., Millar, J. K., Devon, R. S., Blackwood, D. & Muir, W. J. (2004). A genome scan and follow-up study identify a bipolar disorder susceptibility locus on chromosome 1q42. ***Mol Psychiatry*** **9, 1083-1090.**

Machado-Vieira, R., Andreazza, A. C., Viale, C. I., Zanatto, V., Cereser, V., Jr., da, S., V, Kapczinski, F., Portela, L. V., Souza, D. O., Salvador, M. & Gentil, V. (2007). Oxidative stress parameters in unmedicated and treated bipolar subjects during initial manic episode: a possible role for lithium antioxidant effects. ***Neurosci. Lett.*** **421, 33-36.**

Machado-Vieira, R., Lara, D. R., Portela, L. V., Goncalves, C. A., Soares, J. C., Kapczinski, F. & Souza, D. O. (2002). Elevated serum S100B protein in drug-free bipolar patients during first manic episode: a pilot study. ***Eur. Neuropsychopharmacol.*** **12, 269-272.**

MacIntyre, D. J., Blackwood, D. H., Porteous, D. J., Pickard, B. S. & Muir, W. J. (2003). Chromosomal abnormalities and mental illness. *Mol Psychiatry* 8, 275-287.

Maeda, K., Nwulia, E., Chang, J., Balkissoon, R., Ishizuka, K., Chen, H., Zandi, P., McInnis, M. G. & Sawa, A. (2006). Differential expression of disrupted-in-schizophrenia (DISC1) in bipolar disorder. *Biol. Psychiatry* 60, 929-935.

Maes, M., Bosmans, E., Calabrese, J., Smith, R. & Meltzer, H. Y. (1995a). Interleukin-2 and interleukin-6 in schizophrenia and mania: effects of neuroleptics and mood stabilizers. *J Psychiatr. Res.* 29, 141-152.

Maes, M., Delange, J., Ranjan, R., Meltzer, H. Y., Desnyder, R., Cooremans, W. & Scharpe, S. (1997). Acute phase proteins in schizophrenia, mania and major depression: modulation by psychotropic drugs. *Psychiatry Res.* 66, 1-11.

Maes, M., Meltzer, H. Y., Buckley, P. & Bosmans, E. (1995b). Plasma-soluble interleukin-2 and transferrin receptor in schizophrenia and major depression. *Eur. Arch. Psychiatry Clin. Neurosci.* 244, 325-329.

Malaspina, D. (2001). Paternal factors and schizophrenia risk: de novo mutations and imprinting. *Schizophr. Bull.* 27, 379-393.

Man, E. B., Bettcher, P. G. & . (1947a). Plasma-amino acids, serum lipids, and proteins after prefrontal lobotomy in seven schizophrenic patients. *Yale J Biol. Med.* 20, 175-183.

Man, E. B., Bettcher, P. G. & Brown, W. T. (1947b). Variations in plasma-amino acid nitrogen of schizophrenics. *Yale J Biol. Med.* 20, 167-174.

Manolio, T. A., Collins, F. S., Cox, N. J., Goldstein, D. B., Hindorff, L. A., Hunter, D. J., McCarthy, M. I., Ramos, E. M., Cardon, L. R., Chakravarti, A., Cho, J. H., Guttmacher, A. E., Kong, A., Kruglyak, L., Mardis, E., Rotimi, C. N., Slatkin, M., Valle, D., Whittemore, A. S., Boehnke, M., Clark, A. G., Eichler, E. E., Gibson, G., Haines, J. L., Mackay, T. F., McCarroll, S. A. & Visscher, P. M. (2009). Finding the missing heritability of complex diseases. *Nature* 461, 747-753.

Mao, Y., Ge, X., Frank, C. L., Madison, J. M., Koehler, A. N., Doud, M. K., Tassa, C., Berry, E. M., Soda, T., Singh, K. K., Biechele, T., Petryshen, T. L., Moon, R. T., Haggarty, S. J. & Tsai, L. H. (2009). Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3beta/beta-catenin signaling. *Cell* 136, 1017-1031.

Marrazzo, A., Fiorito, J., Zappala, L., Prezzavento, O., Ronsisvalle, S., Pasquinucci, L., Scoto, G. M., Bernardini, R. & Ronsisvalle, G. (2011). Antiproliferative activity of phenylbutyrate ester of haloperidol metabolite II [(+/-)-MRJF4] in prostate cancer cells. *Eur. J Med. Chem.* 46, 433-438.

Martins-De-Souza, D., Gattaz, W. F., Schmitt, A., Novello, J. C., Marangoni, S., Turck, C. W. & as-Neto, E. (2009a). Proteome analysis of schizophrenia patients Wernicke's area reveals an energy metabolism dysregulation. *BMC. Psychiatry* 9, 17.

Martins-De-Souza, D., Gattaz, W. F., Schmitt, A., Rewerts, C., Marangoni, S., Novello, J. C., Maccarrone, G., Turck, C. W. & as-Neto, E. (2009b). Alterations in oligodendrocyte proteins, calcium homeostasis and new potential markers in schizophrenia anterior temporal lobe are revealed by shotgun proteome analysis. *J Neural Transm.* 116, 275-289.

Martins-De-Souza, D., Lebar, M. & Turck, C. W. (2011). Proteome analyses of cultured astrocytes treated with MK-801 and clozapine: similarities with schizophrenia. ***Eur. Arch. Psychiatry Clin. Neurosci.*** **261, 217-228.**

Martins-De-Souza, D., Maccarrone, G., Wobrock, T., Zerr, I., Gormanns, P., Reckow, S., Falkai, P., Schmitt, A. & Turck, C. W. (2010). Proteome analysis of the thalamus and cerebrospinal fluid reveals glycolysis dysfunction and potential biomarkers candidates for schizophrenia. ***J Psychiatr. Res.*** **44, 1176-1189.**

Mashayekhi, F. & Salehin, Z. (2006). Cerebrospinal fluid nerve growth factor levels in patients with Alzheimer's disease. ***Ann. Saudi. Med.*** **26, 278-282.**

Mateos, S. S., Sanchez, C. L., Paredes, S. D., Barriga, C. & Rodriguez, A. B. (2009). Circadian levels of serotonin in plasma and brain after oral administration of tryptophan in rats. ***Basic Clin. Pharmacol. Toxicol.*** **104, 52-59.**

Mathur, A., Law, M. H., Megson, I. L., Shaw, D. J. & Wei, J. (2010). Genetic association of the AKT1 gene with schizophrenia in a British population. ***Psychiatr. Genet.*** **20, 118-122.**

Mauri, M. C., Rudelli, R., Vanni, S., Panza, G., Sicaro, A., Audisio, D., Sacerdote, P. & Panerai, A. E. (1998). Cholecystokinin, beta-endorphin and vasoactive intestinal peptide in peripheral blood mononuclear cells of drug-naive schizophrenic patients treated with haloperidol compared to healthy controls. ***Psychiatry Res.*** **78, 45-50.**

Mazurek, S. (2011). Pyruvate kinase type M2: a key regulator of the metabolic budget system in tumor cells. ***Int. J Biochem. Cell Biol.*** **43, 969-980.**

McAllister, C. G., van Kammen, D. P., Rehn, T. J., Miller, A. L., Gurklis, J., Kelley, M. E., Yao, J. & Peters, J. L. (1995). Increases in CSF levels of interleukin-2 in schizophrenia: effects of recurrence of psychosis and medication status. ***Am J Psychiatry*** **152, 1291-1297.**

McGeer, P. L., McGeer, E. G. & Boulding, J. E. (1956). Relation of aromatic amino acids to excretory pattern of schizophrenics. ***Science*** **123, 1078-1080.**

McGrath, J., Saha, S., Welham, J., El, S. O., MacCauley, C. & Chant, D. (2004). A systematic review of the incidence of schizophrenia: the distribution of rates and the influence of sex, urbanicity, migrant status and methodology. ***BMC. Med.*** **2, 13.**

McManus, C. A., Polden, J., Cotter, D. R. & Dunn, M. J. (2010). Two-dimensional reference map for the basic proteome of the human dorsolateral prefrontal cortex (dlPFC) of the prefrontal lobe region of the brain. ***Proteomics.*** **10, 2551-2555.**

Mead, C. L., Kuzyk, M. A., Moradian, A., Wilson, G. M., Holt, R. A. & Morin, G. B. (2010). Cytosolic protein interactions of the schizophrenia susceptibility gene dysbindin. ***J Neurochem.*** **113, 1491-1503.**

Medina-Hernandez, V., Ramos-Loyo, J., Luquin, S., Sanchez, L. F., Garcia-Estrada, J. & Navarro-Ruiz, A. (2007). Increased lipid peroxidation and neuron specific enolase in treatment refractory schizophrenics. ***J Psychiatr. Res.*** **41, 652-658.**

Mednick, S. A., Machon, R. A., Huttunen, M. O. & Bonett, D. (1988). Adult schizophrenia following prenatal exposure to an influenza epidemic. ***Arch. Gen. Psychiatry*** **45, 189-192.**

Mellsop, G. W., Koadlow, L., Syme, J. & Whittingham, S. (1974). Absence of rheumatoid arthritis in schizophrenia. ***Aust. N. Z. J Med.*** **4, 247-252.**

Middleton, F. A., Pato, C. N., Gentile, K. L., McGann, L., Brown, A. M., Trauzzi, M., Diab, H., Morley, C. P., Medeiros, H., Macedo, A., Azevedo, M. H. & Pato, M. T. (2005). Gene expression analysis of peripheral blood leukocytes from discordant sib-pairs with schizophrenia and bipolar disorder reveals points of convergence between genetic and functional genomic approaches. ***Am J Med Genet B Neuropsychiatr Genet*** **136B.**

Mildvan, D., Landay, A., De Gruttola, V., Machado, S. G. & Kagan, J. (1997). An approach to the validation of markers for use in AIDS clinical trials. ***Clin Infect Dis*** **24.**

Millar, J. K., Pickard, B. S., Mackie, S., James, R., Christie, S., Buchanan, S. R., Malloy, M. P., Chubb, J. E., Huston, E., Baillie, G. S., Thomson, P. A., Hill, E. V., Brandon, N. J., Rain, J. C., Camargo, L. M., Whiting, P. J., Houslay, M. D., Blackwood, D. H., Muir, W. J. & Porteous, D. J. (2005). DISC1 and PDE4B are interacting genetic factors in schizophrenia that regulate cAMP signaling. ***Science*** **310, 1187-1191.**

Millar, J. K., Wilson-Annan, J. C., Anderson, S., Christie, S., Taylor, M. S., Semple, C. A., Devon, R. S., St Clair, D. M., Muir, W. J., Blackwood, D. H. & Porteous, D. J. (2000). Disruption of two novel genes by a translocation co-segregating with schizophrenia. ***Hum. Mol Genet.*** **9, 1415-1423.**

Miller, C. L., Llenos, I. C., Dulay, J. R., Barillo, M. M., Yolken, R. H. & Weis, S. (2004). Expression of the kynurenine pathway enzyme tryptophan 2,3-dioxygenase is increased in the frontal cortex of individuals with schizophrenia. ***Neurobiol. Dis.*** **15, 618-629.**

Miners, S., Ashby, E., Baig, S., Harrison, R., Tayler, H., Speedy, E., Prince, J. A., Love, S. & Kehoe, P. G. (2009). Angiotensin-converting enzyme levels and activity in Alzheimer's disease: differences in brain and CSF ACE and association with ACE1 genotypes. ***Am J Transl. Res.*** **1, 163-177.**

Mirnics, K., Middleton, F. A., Lewis, D. A. & Levitt, P. (2001). Analysis of complex brain disorders with gene expression microarrays: schizophrenia as a disease of the synapse. ***Trends Neurosci.*** **24, 479-486.**

Mirnics, K., Middleton, F. A., Marquez, A., Lewis, D. A. & Levitt, P. (2000). Molecular characterization of schizophrenia viewed by microarray analysis of gene expression in prefrontal cortex. ***Neuron*** **28, 53-67.**

Mitkus, S. N., Hyde, T. M., Vakkalanka, R., Kolachana, B., Weinberger, D. R., Kleinman, J. E. & Lipska, B. K. (2008). Expression of oligodendrocyte-associated genes in dorsolateral prefrontal cortex of patients with schizophrenia. ***Schizophr. Res.*** **98, 129-138.**

Mogi, M., Harada, M., Narabayashi, H., Inagaki, H., Minami, M. & Nagatsu, T. (1996). Interleukin (IL)-1 beta, IL-2, IL-4, IL-6 and transforming growth factor-alpha levels are elevated in ventricular cerebrospinal fluid in juvenile parkinsonism and Parkinson's disease. ***Neurosci. Lett.*** **211, 13-16.**

Mohamed, S. N., Merskey, H., Kazarian, S. & Disney, T. F. (1982). An investigation of the possible inverse relationships between the occurrence of rheumatoid arthritis, osteoarthritis and schizophrenia. ***Can. J Psychiatry*** **27, 381-383.**

Moncrieff, J. & Leo, J. (2010). A systematic review of the effects of antipsychotic drugs on brain volume. *Psychol. Med.* 40, 1409-1422.

Monji, A., Kato, T. & Kanba, S. (2009). Cytokines and schizophrenia: Microglia hypothesis of schizophrenia. *Psychiatry Clin. Neurosci.* 63, 257-265.

Morgan, C., Kirkbride, J., Leff, J., Craig, T., Hutchinson, G., McKenzie, K., Morgan, K., Dazzan, P., Doody, G. A., Jones, P., Murray, R. & Fearon, P. (2007). Parental separation, loss and psychosis in different ethnic groups: a case-control study. *Psychol. Med.* 37, 495-503.

Morgan, M. S. & Pilgrim, F. J. (1952). Concentration of hyperglycemic factor from urine of schizophrenics. *Proc. Soc. Exp. Biol. Med.* 79, 106-111.

Morris, H. R., Paxton, T., Dell, A., Langhorne, J., Berg, M., Bordoli, R. S., Hoyes, J. & Bateman, R. H. (1996). High sensitivity collisionally-activated decomposition tandem mass spectrometry on a novel quadrupole/orthogonal-acceleration time-of-flight mass spectrometer. *Rapid Commun. Mass Spectrom.* 10, 889-896.

Mors, O., Ewald, H., Blackwood, D. & Muir, W. (1997). Cytogenetic abnormalities on chromosome 18 associated with bipolar affective disorder or schizophrenia. *Br. J Psychiatry* 170, 278-280.

Mrazek, D. A., Biernacka, J. M., O'Kane, D. J., Black, J. L., Cunningham, J. M., Drews, M. S., Snyder, K. A., Stevens, S. R., Rush, A. J. & Weinshilboum, R. M. (2011). CYP2C19 variation and citalopram response. *Pharmacogenet. Genomics* 21, 1-9.

Mudge, J., Miller, N. A., Khrebtukova, I., Lindquist, I. E., May, G. D., Huntley, J. J., Luo, S., Zhang, L., van Velkinburgh, J. C., Farmer, A. D., Lewis, S., Beavis, W. D., Schilkey, F. D., Virk, S. M., Black, C. F., Myers, M. K., Mader, L. C., Langley, R. J., Utsey, J. P., Kim, R. W., Roberts, R. C., Khalsa, S. K., Garcia, M., mbriz-Griffith, V., Harlan, R., Czika, W., Martin, S., Wolfinger, R. D., Perrone-Bizzozero, N. I., Schroth, G. P. & Kingsmore, S. F. (2008). Genomic convergence analysis of schizophrenia: mRNA sequencing reveals altered synaptic vesicular transport in post-mortem cerebellum. *PLoS. One.* 3, e3625.

Muller, N., Dobmeier, P., Empl, M., Riedel, M., Schwarz, M. & Ackenheil, M. (1997). Soluble IL-6 receptors in the serum and cerebrospinal fluid of paranoid schizophrenic patients. *Eur. Psychiatry* 12, 294-299.

Muller, N., Krause, D., Dehning, S., Musil, R., Schennach-Wolff, R., Obermeier, M., Moller, H. J., Klauss, V., Schwarz, M. J. & Riedel, M. (2010). Celecoxib treatment in an early stage of schizophrenia: results of a randomized, double-blind, placebo-controlled trial of celecoxib augmentation of amisulpride treatment. *Schizophr. Res.* 121, 118-124.

Muller, N. & Schwarz, M. (2006). Schizophrenia as an inflammation-mediated dysbalance of glutamatergic neurotransmission. *Neurotox. Res.* 10, 131-148.

Muller, T., Blum-Degen, D., Przuntek, H. & Kuhn, W. (1998). Interleukin-6 levels in cerebrospinal fluid inversely correlate to severity of Parkinson's disease. *Acta Neurol. Scand.* 98, 142-144.

Murphy, B. P., Chung, Y. C., Park, T. W. & McGorry, P. D. (2006). Pharmacological treatment of primary negative symptoms in schizophrenia: a systematic review. ***Schizophr. Res.*** **88, 5-25.**

Murray, R. M. & Lewis, S. W. (1987). Is schizophrenia a neurodevelopmental disorder? ***Br. Med. J (Clin. Res. Ed)*** **295, 681-682.**

Na, K. S. & Kim, Y. K. (2007). Monocytic, Th1 and th2 cytokine alterations in the pathophysiology of schizophrenia. ***Neuropsychobiology*** **56, 55-63.**

Nadri, C., Kozlovsky, N., Agam, G. & Bersudsky, Y. (2002). GSK-3 parameters in lymphocytes of schizophrenic patients. ***Psychiatry Res.*** **112, 51-57.**

Nagai, T., Ibi, D. & Yamada, K. (2011). Animal model for schizophrenia that reflects gene-environment interactions. ***Biol. Pharm. Bull.*** **34, 1364-1368.**

Nagao, M. & Hayashi, H. (2009). Glycogen synthase kinase-3beta is associated with Parkinson's disease. ***Neurosci. Lett.*** **449, 103-107.**

Nakki, R., Koistinaho, J., Sharp, F. R. & Sagar, S. M. (1995). Cerebellar toxicity of phencyclidine. ***J Neurosci.*** **15, 2097-2108.**

Narayan, S., Tang, B., Head, S. R., Gilmartin, T. J., Sutcliffe, J. G., Dean, B. & Thomas, E. A. (2008). Molecular profiles of schizophrenia in the CNS at different stages of illness. ***Brain Res.*** **1239, 235-248.**

Nasr, S., Altman, E. G. & Meltzer, H. Y. (1981). Concordance of atopic and affective disorders. ***J Affect. Disord.*** **3, 291-296.**

Natesan, S., Reckless, G. E., Barlow, K. B., Nobrega, J. N. & Kapur, S. (2008). Amisulpride the 'atypical' atypical antipsychotic--comparison to haloperidol, risperidone and clozapine. ***Schizophr. Res.*** **105, 224-235.**

Naudin, J., Mege, J. L., Azorin, J. M. & Dassa, D. (1996). Elevated circulating levels of IL-6 in schizophrenia. ***Schizophr. Res.*** **20, 269-273.**

Navari, S. & Dazzan, P. (2009). Do antipsychotic drugs affect brain structure? A systematic and critical review of MRI findings. ***Psychol. Med.*** **39, 1763-1777.**

Nedjadi, T., Kitteringham, N., Campbell, F., Jenkins, R. E., Park, B. K., Navarro, P., Ashcroft, F., Tepikin, A., Neoptolemos, J. P. & Costello, E. (2009). S100A6 binds to annexin 2 in pancreatic cancer cells and promotes pancreatic cancer cell motility. ***Br. J Cancer*** **101, 1145-1154.**

Nesvizhskii, A. I., Keller, A., Kolker, E. & Aebersold, R. (2003). A statistical model for identifying proteins by tandem mass spectrometry. ***Anal. Chem.*** **75, 4646-4658.**

Nigro, P., Satoh, K., O'Dell, M. R., Soe, N. N., Cui, Z., Mohan, A., Abe, J., Alexis, J. D., Sparks, J. D. & Berk, B. C. (2011). Cyclophilin A is an inflammatory mediator that promotes atherosclerosis in apolipoprotein E-deficient mice. ***J Exp. Med.*** **208, 53-66.**

Niwa, M., Kamiya, A., Murai, R., Kubo, K., Gruber, A. J., Tomita, K., Lu, L., Tomisato, S., Jaaro-Peled, H., Seshadri, S., Hiyama, H., Huang, B., Kohda, K., Noda, Y., O'Donnell, P., Nakajima, K., Sawa, A. & Nabeshima, T. (2010). Knockdown of DISC1 by in utero gene

transfer disturbs postnatal dopaminergic maturation in the frontal cortex and leads to adult behavioral deficits. *Neuron* 65, 480-489.

Nobeli, I. & Thornton, J. M. (2006). A bioinformatician's view of the metabolome. *Bioessays* 28, 534-545.

Norman, R. M. & Malla, A. K. (1993). Stressful life events and schizophrenia. I: A review of the research. *Br. J Psychiatry* 162, 161-166.

Norton, N., Williams, H. J., Dwyer, S., Carroll, L., Peirce, T., Moskvina, V., Segurado, R., Nikolov, I., Williams, N. M., Ikeda, M., Iwata, N., Owen, M. J. & O'Donovan, M. C. (2007). Association analysis of AKT1 and schizophrenia in a UK case control sample. *Schizophr. Res.* 93, 58-65.

Novikova, S. I., He, F., Cutrufello, N. J. & Lidow, M. S. (2006). Identification of protein biomarkers for schizophrenia and bipolar disorder in the postmortem prefrontal cortex using SELDI-TOF-MS ProteinChip profiling combined with MALDI-TOF-PSD-MS analysis. *Neurobiol. Dis.* 23, 61-76.

Numata, S., Ueno, S., Iga, J., Yamauchi, K., Hongwei, S., Hashimoto, R., Takeda, M., Kunugi, H., Itakura, M. & Ohmori, T. (2007). Gene expression in the peripheral leukocytes and association analysis of PDLIM5 gene in schizophrenia. *Neurosci. Lett.* 415, 28-33.

O'Connor, D. T., Cervenka, J. H., Stone, R. A., Parmer, R. J., Franco-Bourland, R. E., Madrazo, I. & Langlais, P. J. (1993). Chromogranin A immunoreactivity in human cerebrospinal fluid: properties, relationship to noradrenergic neuronal activity, and variation in neurologic disease. *Neuroscience* 56, 999-1007.

O'Donovan, M. C., Craddock, N. J. & Owen, M. J. (2009). Genetics of psychosis; insights from views across the genome. *Hum. Genet.* 126, 3-12.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250, 4007-4021.

Oike, Y., Hata, A., Mamiya, T., Kaname, T., Noda, Y., Suzuki, M., Yasue, H., Nabeshima, T., Araki, K. & Yamamura, K. (1999). Truncated CBP protein leads to classical Rubinstein-Taybi syndrome phenotypes in mice: implications for a dominant-negative mechanism. *Hum. Mol Genet.* 8, 387-396.

Olsson, A., Vanderstichele, H., Andreasen, N., De, M. G., Wallin, A., Holmberg, B., Rosengren, L., Vanmechelen, E. & Blennow, K. (2005). Simultaneous measurement of beta-amyloid(1-42), total tau, and phosphorylated tau (Thr181) in cerebrospinal fluid by the xMAP technology. *Clin. Chem.* 51, 336-345.

Omenn, G. S., States, D. J., Adamski, M., Blackwell, T. W., Menon, R., Hermjakob, H., Apweiler, R., Haab, B. B., Simpson, R. J., Eddes, J. S., Kapp, E. A., Moritz, R. L., Chan, D. W., Rai, A. J., Admon, A., Aebersold, R., Eng, J., Hancock, W. S., Hefta, S. A., Meyer, H., Paik, Y. K., Yoo, J. S., Ping, P., Pounds, J., Adkins, J., Qian, X., Wang, R., Wasinger, V., Wu, C. Y., Zhao, X., Zeng, R., Archakov, A., Tsugita, A., Beer, I., Pandey, A., Pisano, M., Andrews, P., Tammen, H., Speicher, D. W. & Hanash, S. M. (2005). Overview of the HUPO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics.* 5, 3226-3245.

Ono, T., Hashimoto, E., Ukai, W., Ishii, T. & Saito, T. (2010). The role of neural stem cells for in vitro models of schizophrenia: neuroprotection via Akt/ERK signal regulation. *Schizophr. Res.* 122, 239-247.

Ozdemir, V., Fourie, J. & Ozdener, F. (2002). Aripiprazole (Otsuka Pharmaceutical Co). *Curr. Opin. Investig. Drugs* 3, 113-120.

Ozeki, Y., Tomoda, T., Kleiderlein, J., Kamiya, A., Bord, L., Fujii, K., Okawa, M., Yamada, N., Hatten, M. E., Snyder, S. H., Ross, C. A. & Sawa, A. (2003). Disrupted-in-Schizophrenia-1 (DISC-1): mutant truncation prevents binding to NudE-like (NUDEL) and inhibits neurite outgrowth. *Proc. Natl. Acad. Sci. U. S. A* 100, 289-294.

Palagi, P. M., Hernandez, P., Walther, D. & Appel, R. D. (2006). Proteome informatics I: bioinformatics tools for processing experimental data. *Proteomics.* 6, 5435-5444.

Palomino, A., Vallejo-Illarramendi, A., Gonzalez-Pinto, A., Aldama, A., Gonzalez-Gomez, C., Mosquera, F., Gonzalez-Garcia, G. & Matute, C. (2006). Decreased levels of plasma BDNF in first-episode schizophrenia and bipolar disorder patients. *Schizophr. Res.* 86, 321-322.

Pan, Q., Shai, O., Lee, L. J., Frey, B. J. & Blencowe, B. J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* 40, 1413-1415.

Panza, G., Monzani, E., Sacerdote, P., Penati, G. & Panerai, A. E. (1992). Beta-endorphin, vasoactive intestinal peptide and cholecystokinin in peripheral blood mononuclear cells from healthy subjects and from drug-free and haloperidol-treated schizophrenic patients. *Acta Psychiatr. Scand.* 85, 207-210.

Park, Y. U., Jeong, J., Lee, H., Mun, J. Y., Kim, J. H., Lee, J. S., Nguyen, M. D., Han, S. S., Suh, P. G. & Park, S. K. (2010). Disrupted-in-schizophrenia 1 (DISC1) plays essential roles in mitochondria in collaboration with Mitofilin. *Proc. Natl. Acad. Sci. U. S. A* 107, 17785-17790.

Pedersen, C. B. & Mortensen, P. B. (2001). Evidence of a dose-response relationship between urbanicity during upbringing and schizophrenia risk. *Arch. Gen. Psychiatry* 58, 1039-1046.

Pedrini, M., Chendo, I., Grande, I., Lobato, M. I., Belmonte-de-Abreu, P. S., Lersch, C., Walz, J., Kauer-Santanna, M., Kapczinski, F. & Gama, C. S. (2011). SERUM BRAIN-DERIVED NEUROTROPHIC FACTOR AND CLOZAPINE DAILY DOSE IN PATIENTS WITH SCHIZOPHRENIA: A POSITIVE CORRELATION. *Neurosci. Lett.*

Pekary, A. E., Gwirtsman, H., Tourtellotte, W. W., Smith, V. P. & Hershman, J. M. (1991). High levels of thyrotropin-releasing hormone precursor peptide immunoreactivity and binding substance occur in human cerebrospinal fluid. *Neuroendocrinology* 53, 246-252.

Penner, J. D. & Brown, A. S. (2007). Prenatal infectious and nutritional factors and risk of adult schizophrenia. *Expert. Rev. Neurother.* 7, 797-805.

Perez-Neri, I., Ramirez-Bermudez, J., Montes, S. & Rios, C. (2006). Possible mechanisms of neurodegeneration in schizophrenia. *Neurochem. Res.* 31, 1279-1294.

Perkins, D. O., Jeffries, C. D., Jarskog, L. F., Thomson, J. M., Woods, K., Newman, M. A., Parker, J. S., Jin, J. & Hammond, S. M. (2007). microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. *Genome Biol.* 8, R27.

Perl, O., Ilani, T., Strous, R. D., Lapidus, R. & Fuchs, S. (2003). The alpha7 nicotinic acetylcholine receptor in schizophrenia: decreased mRNA levels in peripheral blood lymphocytes. *FASEB J.* 17, 1948-1950.

Perrone-Bizzozero, N. I., Sower, A. C., Bird, E. D., Benowitz, L. I., Ivins, K. J. & Neve, R. L. (1996). Levels of the growth-associated protein GAP-43 are selectively increased in association cortices in schizophrenia. *Proc. Natl. Acad. Sci. U. S. A* 93, 14182-14187.

Perroud, N., Uher, R., Ng, M. Y., Guipponi, M., Hauser, J., Henigsberg, N., Maier, W., Mors, O., Gennarelli, M., Rietschel, M., Souery, D., Dernovsek, M. Z., Stamp, A. S., Lathrop, M., Farmer, A., Breen, G., Aitchison, K. J., Lewis, C. M., Craig, I. W. & McGuffin, P. (2010). Genome-wide association study of increasing suicidal ideation during antidepressant treatment in the GENDEP project. *Pharmacogenomics. J* .

Peskind, E. R., Griffin, W. S., Akama, K. T., Raskind, M. A. & Van Eldik, L. J. (2001). Cerebrospinal fluid S100B is elevated in the earlier stages of Alzheimer's disease. *Neurochem. Int.* 39, 409-413.

Petersen, A., Gil, J., Maat-Schieman, M. L., Bjorkqvist, M., Tanila, H., Araujo, I. M., Smith, R., Popovic, N., Wierup, N., Norlen, P., Li, J. Y., Roos, R. A., Sundler, F., Mulder, H. & Brundin, P. (2005). Orexin loss in Huntington's disease. *Hum. Mol Genet.* 14, 39-47.

Petzold, A., Jenkins, R., Watt, H. C., Green, A. J., Thompson, E. J., Keir, G., Fox, N. C. & Rossor, M. N. (2003). Cerebrospinal fluid S100B correlates with brain atrophy in Alzheimer's disease. *Neurosci. Lett.* 336, 167-170.

Picchioni, M. M. & Murray, R. M. (2007). Schizophrenia. *BMJ* 335, 91-95.

Pieper, R., Gatlin, C. L., Makusky, A. J., Russo, P. S., Schatz, C. R., Miller, S. S., Su, Q., McGrath, A. M., Estock, M. A., Parmar, P. P., Zhao, M., Huang, S. T., Zhou, J., Wang, F., Esquer-Blasco, R., Anderson, N. L., Taylor, J. & Steiner, S. (2003). The human serum proteome: display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. *Proteomics.* 3, 1345-1364.

Pieper, R., Gatlin, C. L., McGrath, A. M., Makusky, A. J., Mondal, M., Seonarain, M., Field, E., Schatz, C. R., Estock, M. A., Ahmed, N., Anderson, N. G. & Steiner, S. (2004). Characterization of the human urinary proteome: a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. *Proteomics.* 4, 1159-1174.

Pierga, J. Y., Reis-Filho, J. S., Cleator, S. J., Dexter, T., Mackay, A., Simpson, P., Fenwick, K., Iravani, M., Salter, J., Hills, M., Jones, C., Ashworth, A., Smith, I. E., Powles, T. & Dowsett, M. (2007). Microarray-based comparative genomic hybridisation of breast cancer patients receiving neoadjuvant chemotherapy. *Br. J Cancer* 96, 341-351.

Pillai, A., Kale, A., Joshi, S., Naphade, N., Raju, M. S., Nasrallah, H. & Mahadik, S. P. (2010). Decreased BDNF levels in CSF of drug-naive first-episode psychotic subjects: correlation with plasma BDNF and psychopathology. *Int. J Neuropsychopharmacol.* 13, 535-539.

Pillai, A., Veeranan-Karmegam, R., Dhandapani, K. M. & Mahadik, S. P. (2008). Cystamine prevents haloperidol-induced decrease of BDNF/TrkB signaling in mouse frontal cortex. ***J Neurochem.*** **107, 941-951.**

Poduslo, S. E. (1993). A new polymorphism in the gene for GAP43. ***Hum. Genet.*** **92, 635-636.**

Pollock, K., Stroemer, P., Patel, S., Stevanato, L., Hope, A., Miljan, E., Dong, Z., Hodges, H., Price, J. & Sinden, J. D. (2006). A conditionally immortal clonal stem cell line from human cortical neuroepithelium for the treatment of ischemic stroke. ***Exp. Neurol.*** **199, 143-155.**

Poltorak, M., Wright, R., Hemperly, J. J., Torrey, E. F., Issa, F., Wyatt, R. J. & Freed, W. J. (1997). Monozygotic twins discordant for schizophrenia are discordant for N-CAM and L1 in CSF. ***Brain Res.*** **751, 152-154.**

Poon, H. F., Hensley, K., Thongboonkerd, V., Merchant, M. L., Lynn, B. C., Pierce, W. M., Klein, J. B., Calabrese, V. & Butterfield, D. A. (2005). Redox proteomics analysis of oxidatively modified proteins in G93A-SOD1 transgenic mice--a model of familial amyotrophic lateral sclerosis. ***Free Radic. Biol. Med.*** **39, 453-462.**

Prabakaran, S., Swatton, J. E., Ryan, M. M., Huffaker, S. J., Huang, J. T., Griffin, J. L., Wayland, M., Freeman, T., Dudbridge, F., Lilley, K. S., Karp, N. A., Hester, S., Tkachev, D., Mimmack, M. L., Yolken, R. H., Webster, M. J., Torrey, E. F. & Bahn, S. (2004). Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. ***Mol Psychiatry*** **9, 684-97, 643.**

Prabakaran, S., Wengenroth, M., Lockstone, H. E., Lilley, K., Leweke, F. M. & Bahn, S. (2007). 2-D DIGE analysis of liver and red blood cells provides further evidence for oxidative stress in schizophrenia. ***J Proteome Res*** **6.**

Prentice, R. L. (1989). Surrogate endpoints in clinical trials: definition and operational criteria. ***Stat Med*** **8.**

Pulford, D. J., Adams, F., Henry, B., Mallinson, D. J., Reid, I. C. & Stewart, C. A. (2006). Chronic lithium administration down regulates transthyretin mRNA expression in rat choroid plexus. ***Neuropsychiatr. Dis. Treat.*** **2, 549-555.**

Quelo, I. & Jurdic, P. (2000). Differential regulation of the carbonic anhydrase II gene expression by hormonal nuclear receptors in monocytic cells: identification of the retinoic acid response element. ***Biochem. Biophys. Res. Commun.*** **271, 481-491.**

Quincozes-Santos, A., Bobermin, L. D., Tonial, R. P., Bambini-Junior, V., Riesgo, R. & Gottfried, C. (2010). Effects of atypical (risperidone) and typical (haloperidol) antipsychotic agents on astroglial functions. ***Eur. Arch. Psychiatry Clin. Neurosci.*** **260, 475-481.**

Read, J., van, O. J., Morrison, A. P. & Ross, C. A. (2005). Childhood trauma, psychosis and schizophrenia: a literature review with theoretical and clinical implications. ***Acta Psychiatr. Scand.*** **112, 330-350.**

Reddy, P. H., Mani, G., Park, B. S., Jacques, J., Murdoch, G., Whetsell, W., Jr., Kaye, J. & Manczak, M. (2005). Differential loss of synaptic proteins in Alzheimer's disease: implications for synaptic dysfunction. ***J Alzheimers. Dis.*** **7, 103-117.**

Rezin, G. T., Amboni, G., Zugno, A. I., Quevedo, J. & Streck, E. L. (2009). Mitochondrial dysfunction and psychiatric disorders. ***Neurochem. Res.*** **34, 1021-1029.**

Riedel, M., Strassnig, M., Schwarz, M. J. & Muller, N. (2005). COX-2 inhibitors as adjunctive therapy in schizophrenia: rationale for use and evidence to date. ***CNS. Drugs*** **19, 805-819.**

Righetti, P. G., Castagna, A., Antonucci, F., Piubelli, C., Cecconi, D., Campostrini, N., Rustichelli, C., Antonioli, P., Zanusso, G., Monaco, S., Lomas, L. & Boschetti, E. (2005). Proteome analysis in the clinical chemistry laboratory: myth or reality? ***Clin. Chim. Acta*** **357, 123-139.**

Roche, S., Cassidy, F., Zhao, C., Badger, J., Claffey, E., Mooney, L., Delaney, C., Dobrin, S. & McKeon, P. (2007). Candidate gene analysis of 21q22: support for S100B as a susceptibility gene for bipolar affective disorder with psychosis. ***Am J Med. Genet. B Neuropsychiatr. Genet.*** **144B, 1094-1096.**

Rolan, P. (1997). The contribution of clinical pharmacology surrogates and models to drug development--a critical appraisal. ***Br J Clin Pharmacol*** **44.**

Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A. & Pappin, D. J. (2004). Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. ***Mol. Cell Proteomics.*** **3, 1154-1169.**

Rothermundt, M., Falkai, P., Ponath, G., Abel, S., Burkle, H., Diedrich, M., Hetzel, G., Peters, M., Siegmund, A., Pedersen, A., Maier, W., Schramm, J., Suslow, T., Ohrmann, P. & Arolt, V. (2004). Glial cell dysfunction in schizophrenia indicated by increased S100B in the CSF. ***Mol Psychiatry*** **9, 897-899.**

Ruano, D., Aulchenko, Y. S., Macedo, A., Soares, M. J., Valente, J., Azevedo, M. H., Hutz, M. H., Gama, C. S., Lobato, M. I., Belmonte-de-Abreu, P., Goodman, A. B., Pato, C., Heutink, P. & Palha, J. A. (2008). Association of the gene encoding neurogranin with schizophrenia in males. ***J Psychiatr. Res.*** **42, 125-133.**

Saetre, P., Agartz, I., De, F. A., Lundmark, P., Djurovic, S., Kahler, A., Andreassen, O. A., Jakobsen, K. D., Rasmussen, H. B., Werge, T., Hall, H., Terenius, L. & Jonsson, E. G. (2008). Association between a disrupted-in-schizophrenia 1 (DISC1) single nucleotide polymorphism and schizophrenia in a combined Scandinavian case-control sample. ***Schizophr. Res.*** **106, 237-241.**

Saetre, P., Emilsson, L., Axelsson, E., Kreuger, J., Lindholm, E. & Jazin, E. (2007). Inflammation-related genes up-regulated in schizophrenia brains. ***BMC. Psychiatry*** **7, 46.**

Saha, S., Chant, D., Welham, J. & McGrath, J. (2005). A systematic review of the prevalence of schizophrenia. ***PLoS. Med.*** **2, e141.**

Salehi, Z. & Mashayekhi, F. (2009). Brain-derived neurotrophic factor concentrations in the cerebrospinal fluid of patients with Parkinson's disease. ***J Clin. Neurosci.*** **16, 90-93.**

Samaha, A. N., Seeman, P., Stewart, J., Rajabi, H. & Kapur, S. (2007). "Breakthrough" dopamine supersensitivity during ongoing antipsychotic treatment leads to treatment failure over time. ***J Neurosci.*** **27, 2979-2986.**

Santamaria-Kisiel, L., Rintala-Dempsey, A. C. & Shaw, G. S. (2006). Calcium-dependent and -independent interactions of the S100 protein family. *Biochem. J* 396, 201-214.

Scaglia, F. (2010). The role of mitochondrial dysfunction in psychiatric disease. *Dev. Disabil. Res. Rev.* 16, 136-143.

Scherk, H. & Falkai, P. (2006). Effects of antipsychotics on brain structure. *Curr. Opin. Psychiatry* 19, 145-150.

Schmidt, F. M., Brugel, M., Kratzsch, J., Strauss, M., Sander, C., Baum, P., Thiery, J., Hegerl, U. & Schonknecht, P. (2010). Cerebrospinal fluid hypocretin-1 (orexin A) levels in mania compared to unipolar depression and healthy controls. *Neurosci. Lett.* 483, 20-22.

Schmitt, A., Bertsch, T., Henning, U., Tost, H., Klimke, A., Henn, F. A. & Falkai, P. (2005). Increased serum S100B in elderly, chronic schizophrenic patients: negative correlation with deficit symptoms. *Schizophr. Res.* 80, 305-313.

Schmitt, A., Steyskal, C., Bernstein, H. G., Schneider-Axmann, T., Parlapani, E., Schaeffer, E. L., Gattaz, W. F., Bogerts, B., Schmitz, C. & Falkai, P. (2009). Stereologic investigation of the posterior part of the hippocampus in schizophrenia. *Acta Neuropathol.* 117, 395-407.

Schnieder, T. P. & Dwork, A. J. (2011). Searching for neuropathology: gliosis in schizophrenia. *Biol. Psychiatry* 69, 134-139.

Schosser, A., Gaysina, D., Cohen-Woods, S., Chow, P. C., Martucci, L., Craddock, N., Farmer, A., Korszun, A., Gunasinghe, C., Gray, J., Jones, L., Tozzi, F., Perry, J., Muglia, P., Owen, M. J., Craig, I. W. & McGuffin, P. (2010). Association of DISC1 and TSNAX genes and affective disorders in the depression case-control (DeCC) and bipolar affective case-control (BACCS) studies. *Mol Psychiatry* 15, 844-849.

Schumacher, J., Laje, G., Abou, J. R., Becker, T., Muhleisen, T. W., Vasilescu, C., Mattheisen, M., Herms, S., Hoffmann, P., Hillmer, A. M., Georgi, A., Herold, C., Schulze, T. G., Propping, P., Rietschel, M., McMahon, F. J., Nothen, M. M. & Cichon, S. (2009). The DISC locus and schizophrenia: evidence from an association study in a central European sample and from a meta-analysis across different European populations. *Hum. Mol Genet.* 18, 2719-2727.

Schwarcz, R. & Pellicciari, R. (2002). Manipulation of brain kynurenines: glial targets, neuronal effects, and clinical opportunities. *J Pharmacol. Exp. Ther.* 303, 1-10.

Schwarz, E. & Bahn, S. (2008). The utility of biomarker discovery approaches for the detection of disease mechanisms in psychiatric disorders. *Br J Pharmacol* 153 Suppl 1.

Schwarz, E., Guest, P. C., Rahmoune, H., Harris, L. W., Wang, L., Leweke, F. M., Rothermundt, M., Bogerts, B., Koethe, D., Kranaster, L., Ohrmann, P., Suslow, T., McAllister, G., Spain, M., Barnes, A., van Beveren, N. J., Baron-Cohen, S., Steiner, J., Torrey, F. E., Yolken, R. H. & Bahn, S. (2011a). Identification of a biological signature for schizophrenia in serum. *Mol Psychiatry* .

Schwarz, E., Guest, P. C., Rahmoune, H., Martins-De-Souza, D., Niebuhr, D. W., Weber, N. S., Cowan, D. N., Yolken, R. H., Spain, M., Barnes, A. & Bahn, S. (2011b). Identification of a blood-based biological signature in subjects with psychiatric disorders prior to clinical manifestation. *World J Biol. Psychiatry* .

Sedaghat, F. & Notopoulos, A. (2008). S100 protein family and its application in clinical practice. *Hippokratia.* 12, 198-204.

Selemon, L. D. & Goldman-Rakic, P. S. (1999). The reduced neuropil hypothesis: a circuit based model of schizophrenia. *Biol. Psychiatry* 45, 17-25.

Selemon, L. D., Kleinman, J. E., Herman, M. M. & Goldman-Rakic, P. S. (2002). Smaller frontal gray matter volume in postmortem schizophrenic brains. *Am J Psychiatry* 159, 1983-1991.

Semple, D. M., McIntosh, A. M. & Lawrie, S. M. (2005). Cannabis as a risk factor for psychosis: systematic review. *J Psychopharmacol.* 19, 187-194.

Serafeim, A., Holder, M. J., Grafton, G., Chamba, A., Drayson, M. T., Luong, Q. T., Bunce, C. M., Gregory, C. D., Barnes, N. M. & Gordon, J. (2003). Selective serotonin reuptake inhibitors directly signal for apoptosis in biopsy-like Burkitt lymphoma cells. *Blood* 101, 3212-3219.

Seshadri, S., Kamiya, A., Yokota, Y., Prikulis, I., Kano, S., Hayashi-Takagi, A., Stanco, A., Eom, T. Y., Rao, S., Ishizuka, K., Wong, P., Korth, C., Anton, E. S. & Sawa, A. (2010). Disrupted-in-Schizophrenia-1 expression is regulated by beta-site amyloid precursor protein cleaving enzyme-1-neuregulin cascade. *Proc. Natl. Acad. Sci. U. S. A* 107, 5622-5627.

Sharma, R. P., Martis, B., Rosen, C., Jonalagadda, J., Nemeroff, C. B. & Bissette, G. (2001). CSF thyrotropin-releasing hormone concentrations differ in patients with schizoaffective disorder from patients with schizophrenia or mood disorders. *J Psychiatr. Res.* 35, 287-291.

Shaw, J., Rowlinson, R., Nickson, J., Stone, T., Sweet, A., Williams, K. & Tonge, R. (2003). Evaluation of saturation labelling two-dimensional difference gel electrophoresis fluorescent dyes. *Proteomics.* 3, 1181-1195.

Shen, S., Lang, B., Nakamoto, C., Zhang, F., Pu, J., Kuan, S. L., Chatzi, C., He, S., Mackie, I., Brandon, N. J., Marquis, K. L., Day, M., Hurko, O., McCaig, C. D., Riedel, G. & St, C. D. (2008). Schizophrenia-related neural and behavioral phenotypes in transgenic mice expressing truncated Disc1. *J Neurosci.* 28, 10893-10904.

Sheu, F. S., Azmitia, E. C., Marshak, D. R., Parker, P. J. & Routtenberg, A. (1994). Glial-derived S100b protein selectively inhibits recombinant beta protein kinase C (PKC) phosphorylation of neuron-specific protein F1/GAP43. *Brain Res. Mol Brain Res.* 21, 62-66.

Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* 68, 850-858.

Shi, J., Badner, J. A., Hattori, E., Potash, J. B., Willour, V. L., McMahon, F. J., Gershon, E. S. & Liu, C. (2008). Neurotransmission and bipolar disorder: a systematic family-based association study. *Am J Med. Genet. B Neuropsychiatr. Genet.* 147B, 1270-1277.

Siegel, M. & Tefft, H. (1971). "Pink spot" and its components in normal and schizophrenic urine. *J. Nerv. Ment. Dis.* 152, 412-426.

Simpson, E. H., Kellendonk, C. & Kandel, E. (2010). A possible role for the striatum in the pathogenesis of the cognitive symptoms of schizophrenia. *Neuron* 65, 585-596.

Singh, R. K., Indra, D., Mitra, S., Mondal, R. K., Basu, P. S., Roy, A., Roychowdhury, S. & Panda, C. K. (2007). Deletions in chromosome 4 differentially associated with the development of cervical cancer: evidence of slit2 as a candidate tumor suppressor gene. *Hum. Genet.* 122, 71-81.

Sjogren, M., Minthon, L., Davidsson, P., Granerus, A.-K., Clarberg, A., Vanderstichele, H., Vanmechelen, E., Wallin, A. & Blennow, K. (2000). CSF levels of tau, beta-amyloid(1-42) and GAP-43 in frontotemporal dementia, other types of dementia and normal aging. *J Neural Transm.* 107, 563-579.

Smith, G. S., Workman, C. I., Kramer, E., Hermann, C. R., Ginsberg, R., Ma, Y., Dhawan, V., Chaly, T. & Eidelberg, D. (2011). The relationship between the acute cerebral metabolic response to citalopram and chronic citalopram treatment outcome. *Am J Geriatr. Psychiatry* 19, 53-63.

Sokolov, B. P., Tcherepanov, A. A., Haroutunian, V. & Davis, K. L. (2000). Levels of mRNAs encoding synaptic vesicle and synaptic plasma membrane proteins in the temporal cortex of elderly schizophrenic patients. *Biol. Psychiatry* 48, 184-196.

Sourlingas, T. G., Issidorides, M. R., Alevizos, B., Kontaxakis, V. P., Chrysanthou-Piterou, M., Livaniou, E., Karpouza, A. P. & Sekeri-Pataryas, K. E. (2003). Lymphocytes from bipolar and schizophrenic patients share common biochemical markers related to histone synthesis and histone cell membrane localization characteristic of an activated state. *Psychiatry Res.* 118, 55-67.

Soutar, M. P., Kim, W. Y., Williamson, R., Peggie, M., Hastie, C. J., McLauchlan, H., Snider, W. D., Gordon-Weeks, P. R. & Sutherland, C. (2010). Evidence that glycogen synthase kinase-3 isoforms have distinct substrate preference in the brain. *J Neurochem.* 115, 974-983.

Souza, R. P., Romano-Silva, M. A., Lieberman, J. A., Meltzer, H. Y., Wong, A. H. & Kennedy, J. L. (2008). Association study of GSK3 gene polymorphisms with schizophrenia and clozapine response. *Psychopharmacology (Berl)* 200, 177-186.

Sower, A. C., Bird, E. D. & Perrone-Bizzozero, N. I. (1995). Increased levels of GAP-43 protein in schizophrenic brain tissues demonstrated by a novel immunodetection method. *Mol Chem. Neuropathol.* 24, 1-11.

St, C. D., Blackwood, D., Muir, W., Carothers, A., Walker, M., Spowart, G., Gosden, C. & Evans, H. J. (1990). Association within a family of a balanced autosomal translocation with major mental illness. *Lancet* 336, 13-16.

Stabenau, J. R., Creveling, C. R. & Daly, J. (1970). The "Pink Spot," 3,4-Dimethoxyphenylethylamine, Common Tea, and Schizophrenia. *American Journal of Psychiatry* 127, 611-616.

Steen, R. G., Mull, C., McClure, R., Hamer, R. M. & Lieberman, J. A. (2006). Brain volume in first-episode schizophrenia: systematic review and meta-analysis of magnetic resonance imaging studies. *Br. J Psychiatry* 188, 510-518.

Stefansson, H., Ophoff, R. A., Steinberg, S., Andreassen, O. A., Cichon, S., Rujescu, D., Werge, T., Pietilainen, O. P., Mors, O., Mortensen, P. B., Sigurdsson, E., Gustafsson, O., Nyegaard, M., Tuulio-Henriksson, A., Ingason, A., Hansen, T., Suvisaari, J., Lonnqvist, J., Paunio, T., Borglum, A. D., Hartmann, A., Fink-Jensen, A., Nordentoft, M., Hougaard, D., Norgaard-Pedersen, B., Bottcher, Y., Olesen, J., Breuer, R., Moller, H.

J., Giegling, I., Rasmussen, H. B., Timm, S., Mattheisen, M., Bitter, I., Rethelyi, J. M., Magnusdottir, B. B., Sigmundsson, T., Olason, P., Masson, G., Gulcher, J. R., Haraldsson, M., Fossdal, R., Thorgeirsson, T. E., Thorsteinsdottir, U., Ruggeri, M., Tosato, S., Franke, B., Strengman, E., Kiemeney, L. A., Melle, I., Djurovic, S., Abramova, L., Kaleda, V., Sanjuan, J., de, F. R., Bramon, E., Vassos, E., Fraser, G., Ettinger, U., Picchioni, M., Walker, N., Toulopoulou, T., Need, A. C., Ge, D., Yoon, J. L., Shianna, K. V., Freimer, N. B., Cantor, R. M., Murray, R., Kong, A., Golimbet, V., Carracedo, A., Arango, C., Costas, J., Jonsson, E. G., Terenius, L., Agartz, I., Petursson, H., Nothen, M. M., Rietschel, M., Matthews, P. M., Muglia, P., Peltonen, L., St, C. D., Goldstein, D. B., Stefansson, K. & Collier, D. A. (2009). Common variants conferring risk of schizophrenia. *Nature* 460, 744-747.

Steiner, J., Bielau, H., Brisch, R., Danos, P., Ullrich, O., Mawrin, C., Bernstein, H. G. & Bogerts, B. (2008). Immunological aspects in the neurobiology of suicide: elevated microglial density in schizophrenia and depression is associated with suicide. *J Psychiatr. Res.* 42, 151-157.

Steiner, J., Walter, M., Guest, P., Myint, A. M., Schiltz, K., Panteli, B., Brauner, M., Bernstein, H. G., Gos, T., Herberth, M., Schroeter, M. L., Schwarz, M. J., Westphal, S., Bahn, S. & Bogerts, B. (2010). Elevated S100B levels in schizophrenia are associated with insulin resistance. *Mol Psychiatry* 15, 3-4.

Stevens, J. R. (1982). The neuropathology of schizophrenia. *Psychol. Med.* 12, 695-700.

Stoll, A. L., Renshaw, P. F., Yurgelun-Todd, D. A. & Cohen, B. M. (2000). Neuroimaging in bipolar disorder: what have we learned? *Biol. Psychiatry* 48, 505-517.

Stone, W. S., Faraone, S. V., Su, J., Tarbox, S. I., Van, E. P. & Tsuang, M. T. (2004). Evidence for linkage between regulatory enzymes in glycolysis and schizophrenia in a multiplex sample. *Am J Med. Genet. B Neuropsychiatr. Genet.* 127B, 5-10.

Streck, E. L., Rezin, G. T., Barbosa, L. M., Assis, L. C., Grandi, E. & Quevedo, J. (2007). Effect of antipsychotics on succinate dehydrogenase and cytochrome oxidase activities in rat brain. *Naunyn Schmiedebergs Arch. Pharmacol.* 376, 127-133.

Sugden, M. C. & Holness, M. J. (2002). Therapeutic potential of the mammalian pyruvate dehydrogenase kinases in the prevention of hyperglycaemia. *Curr. Drug Targets. Immune. Endocr. Metabol. Disord.* 2, 151-165.

Sullivan, P. F., Kendler, K. S. & Neale, M. C. (2003). Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. *Arch. Gen. Psychiatry* 60, 1187-1192.

Sunderland, T., Linker, G., Mirza, N., Putnam, K. T., Friedman, D. L., Kimmel, L. H., Bergeson, J., Manetti, G. J., Zimmermann, M., Tang, B., Bartko, J. J. & Cohen, R. M. (2003). Decreased beta-amyloid1-42 and increased tau levels in cerebrospinal fluid of patients with Alzheimer disease. *JAMA* 289, 2094-2103.

Sunderland, T., Mirza, N., Putnam, K. T., Linker, G., Bhupali, D., Durham, R., Soares, H., Kimmel, L., Friedman, D., Bergeson, J., Csako, G., Levy, J. A., Bartko, J. J. & Cohen, R. M. (2004). Cerebrospinal fluid beta-amyloid1-42 and tau in control subjects at risk for Alzheimer's disease: the effect of APOE epsilon4 allele. *Biol. Psychiatry* 56, 670-676.

Sutton, L. P. & Rushlow, W. J. (2011). The effects of neuropsychiatric drugs on glycogen synthase kinase-3 signaling. *Neuroscience* .

Swardfager, W., Lanctot, K., Rothenburg, L., Wong, A., Cappell, J. & Herrmann, N. (2010). A meta-analysis of cytokines in Alzheimer's disease. *Biol. Psychiatry* 68, 930-941.

Syed, F., Rycyzyn, M. A., Westgate, L. & Clevenger, C. V. (2003). A novel and functional interaction between cyclophilin A and prolactin receptor. *Endocrine.* 20, 83-90.

Szabo, S. T., hado-Vieira, R., Yuan, P., Wang, Y., Wei, Y., Falke, C., Cirelli, C., Tononi, G., Manji, H. K. & Du, J. (2009). Glutamate receptors as targets of protein kinase C in the pathophysiology and treatment of animal models of mania. *Neuropharmacology* 56, 47-55.

Szymanski, H. V., Orfanos, A., Narisawa, K., Grosz, R. & Naylor, E. W. (1985). Dihydropteridine reductase in schizophrenic patients. *Psychiatry Res.* 15, 115-119.

Takahashi, N., Ishihara, R., Saito, S., Maemo, N., Aoyama, N., Ji, X., Miura, H., Ikeda, M., Iwata, N., Suzuki, T., Kitajima, T., Yamanouchi, Y., Kinoshita, Y., Ozaki, N. & Inada, T. (2006). Association between chromogranin A gene polymorphism and schizophrenia in the Japanese population. *Schizophr. Res.* 83, 179-183.

Tan, H. Y., Callicott, J. H. & Weinberger, D. R. (2009). Prefrontal cognitive systems in schizophrenia: towards human genetic brain mechanisms. *Cogn Neuropsychiatry* 14, 277-298.

Tan, Y. L., Zhou, D. F. & Zhang, X. Y. (2005). Decreased plasma brain-derived neurotrophic factor levels in schizophrenic patients with tardive dyskinesia: association with dyskinetic movements. *Schizophr. Res.* 74, 263-270.

Tanaka, Y., Yoshida, S., Shimada, Y., Ueda, H. & Asai, K. (2007). Alteration in serum neural cell adhesion molecule in patients of schizophrenia. *Hum. Psychopharmacol.* 22, 97-102.

Tandon, R., Keshavan, M. S. & Nasrallah, H. A. (2008). Schizophrenia, "just the facts" what we know in 2008. 2. Epidemiology and etiology. *Schizophr. Res.* 102, 1-18.

Tascedda, F., Lovati, E., Blom, J. M., Muzzioli, P., Brunello, N., Racagni, G. & Riva, M. A. (1999). Regulation of ionotropic glutamate receptors in the rat brain in response to the atypical antipsychotic seroquel (quetiapine fumarate). *Neuropsychopharmacology* 21, 211-217.

Tatarano, S., Chiyomaru, T., Kawakami, K., Enokida, H., Yoshino, H., Hidaka, H., Yamasaki, T., Kawahara, K., Nishiyama, K., Seki, N. & Nakagawa, M. (2011). miR-218 on the genomic loss region of chromosome 4p15.31 functions as a tumor suppressor in bladder cancer. *Int. J Oncol.* 39, 13-21.

Terry, A. V., Jr., Gearhart, D. A., Mahadik, S. P., Warsi, S., Davis, L. W. & Waller, J. L. (2005). Chronic exposure to typical or atypical antipsychotics in rodents: temporal effects on central alpha7 nicotinic acetylcholine receptors. *Neuroscience* 136, 519-529.

Terry, A. V., Jr., Gearhart, D. A., Warner, S., Hohnadel, E. J., Middlemore, M. L., Zhang, G., Bartlett, M. G. & Mahadik, S. P. (2007a). Protracted effects of chronic oral haloperidol and risperidone on nerve growth factor, cholinergic neurons, and spatial reference learning in rats. *Neuroscience* 150, 413-424.

Terry, A. V., Jr., Gearhart, D. A., Warner, S. E., Zhang, G., Bartlett, M. G., Middlemore, M. L., Beck, W. D., Jr., Mahadik, S. P. & Waller, J. L. (2007b). Oral haloperidol or risperidone

treatment in rats: temporal effects on nerve growth factor receptors, cholinergic neurons, and memory performance. ***Neuroscience*** **146, 1316-1332.**

Thakker-Varia, S., Jean, Y. Y., Parikh, P., Sizer, C. F., Jernstedt, A. J., Parikh, A., Hyde, T. M., Buyske, S. & Alder, J. (2010). The neuropeptide VGF is reduced in human bipolar postmortem brain and contributes to some of the behavioral and molecular effects of lithium. ***J Neurosci.*** **30, 9368-9380.**

Thambisetty, M. & Lovestone, S. (2010). Blood-based biomarkers of Alzheimer's disease: challenging but feasible. ***Biomark. Med.*** **4, 65-79.**

Thambisetty, M., Simmons, A., Velayudhan, L., Hye, A., Campbell, J., Zhang, Y., Wahlund, L. O., Westman, E., Kinsey, A., Guntert, A., Proitsi, P., Powell, J., Causevic, M., Killick, R., Lunnon, K., Lynham, S., Broadstock, M., Choudhry, F., Howlett, D. R., Williams, R. J., Sharp, S. I., Mitchelmore, C., Tunnard, C., Leung, R., Foy, C., O'Brien, D., Breen, G., Furney, S. J., Ward, M., Kloszewska, I., Mecocci, P., Soininen, H., Tsolaki, M., Vellas, B., Hodges, A., Murphy, D. G., Parkins, S., Richardson, J. C., Resnick, S. M., Ferrucci, L., Wong, D. F., Zhou, Y., Muehlboeck, S., Evans, A., Francis, P. T., Spenger, C. & Lovestone, S. (2010a). Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease. ***Arch. Gen. Psychiatry*** **67, 739-748.**

Thambisetty, M., Tripaldi, R., Riddoch-Contreras, J., Hye, A., An, Y., Campbell, J., Sojkova, J., Kinsey, A., Lynham, S., Zhou, Y., Ferrucci, L., Wong, D. F., Lovestone, S. & Resnick, S. M. (2010b). Proteome-based plasma markers of brain amyloid-beta deposition in non-demented older individuals. ***J Alzheimers. Dis.*** **22, 1099-1109.**

Thannickal, T. C., Lai, Y. Y. & Siegel, J. M. (2007). Hypocretin (orexin) cell loss in Parkinson's disease. ***Brain*** **130, 1586-1595.**

Thiede, B., Hohenwarter, W., Krah, A., Mattow, J., Schmid, M., Schmidt, F. & Jungblut, P. R. (2005). Peptide mass fingerprinting. ***Methods*** **35, 237-247.**

Thiselton, D. L., Vladimirov, V. I., Kuo, P. H., McClay, J., Wormley, B., Fanous, A., O'Neill, F. A., Walsh, D., Van den Oord, E. J., Kendler, K. S. & Riley, B. P. (2008). AKT1 is associated with schizophrenia across multiple symptom dimensions in the Irish study of high density schizophrenia families. ***Biol. Psychiatry*** **63, 449-457.**

Thomas, E. A. (2006). Molecular profiling of antipsychotic drug function: convergent mechanisms in the pathology and treatment of psychiatric disorders. ***Mol Neurobiol.*** **34, 109-128.**

Thompson, A., Schafer, J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt, G., Neumann, T., Johnstone, R., Mohammed, A. K. & Hamon, C. (2003a). Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. ***Anal. Chem.*** **75, 1895-1904.**

Thompson, P. M., Kelley, M., Yao, J., Tsai, G. & van Kammen, D. P. (2003b). Elevated cerebrospinal fluid SNAP-25 in schizophrenia. ***Biol. Psychiatry*** **53, 1132-1137.**

Thompson, P. M., Rosenberger, C. & Qualls, C. (1999). CSF SNAP-25 in schizophrenia and bipolar illness. A pilot study. ***Neuropsychopharmacology*** **21, 717-722.**

Thony, B., Auerbach, G. & Blau, N. (2000). Tetrahydrobiopterin biosynthesis, regeneration and functions. ***Biochem. J*** **347 Pt 1, 1-16.**

Thorsell, A., Bjerke, M., Gobom, J., Brunhage, E., Vanmechelen, E., Andreasen, N., Hansson, O., Minthon, L., Zetterberg, H. & Blennow, K. (2010). Neurogranin in cerebrospinal fluid as a marker of synaptic degeneration in Alzheimer's disease. *Brain Res.* 1362, 13-22.

Tian, S. Y., Wang, J. F., Bezchlibnyk, Y. B. & Young, L. T. (2007). Immunoreactivity of 43 kDa growth-associated protein is decreased in post mortem hippocampus of bipolar disorder and schizophrenia. *Neurosci. Lett.* 411, 123-127.

Tienari, P., Wynne, L. C., Sorri, A., Lahti, I., Laksy, K., Moring, J., Naarala, M., Nieminen, P. & Wahlberg, K. E. (2004). Genotype-environment interaction in schizophrenia-spectrum disorder. Long-term follow-up study of Finnish adoptees. *Br. J Psychiatry* 184, 216-222.

Todaro, L., Puricelli, L., Gioseffi, H., Guadalupe, P. M., Lastiri, J., Bal de Kier, J. E., Varela, M. & Sacerdote de, L. E. (2004). Neural cell adhesion molecule in human serum. Increased levels in dementia of the Alzheimer type. *Neurobiol. Dis.* 15, 387-393.

Tonge, R., Shaw, J., Middleton, B., Rowlinson, R., Rayner, S., Young, J., Pognan, F., Hawkins, E., Currie, I. & Davison, M. (2001). Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. *Proteomics.* 1, 377-396.

Tordjman, S., Drapier, D., Bonnot, O., Graignic, R., Fortes, S., Cohen, D., Millet, B., Laurent, C. & Roubertoux, P. L. (2007). Animal models relevant to schizophrenia and autism: validity and limitations. *Behav. Genet.* 37, 61-78.

Torrey, E. F. & Miller, J. (1997). Season of birth and schizophrenia: southern hemisphere data. *Aust. N. Z. J Psychiatry* 31, 308-309.

Tramontina, A. C., Tramontina, F., Bobermin, L. D., Zanotto, C., Souza, D. F., Leite, M. C., Nardin, P., Gottfried, C. & Goncalves, C. A. (2008). Secretion of S100B, an astrocyte-derived neurotrophic protein, is stimulated by fluoxetine via a mechanism independent of serotonin. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 32, 1580-1583.

Tsang, T. M., Huang, J. T., Holmes, E. & Bahn, S. (2006). Metabolic profiling of plasma from discordant schizophrenia twins: correlation between lipid signals and global functioning in female schizophrenia patients. *J Proteome Res* 5.

Uchida, Y., Ohshima, T., Sasaki, Y., Suzuki, H., Yanai, S., Yamashita, N., Nakamura, F., Takei, K., Ihara, Y., Mikoshiba, K., Kolattukudy, P., Honnorat, J. & Goshima, Y. (2005). Semaphorin3A signalling is mediated via sequential Cdk5 and GSK3beta phosphorylation of CRMP2: implication of common phosphorylating mechanism underlying axon guidance and Alzheimer's disease. *Genes Cells* 10, 165-179.

van Berckel, B. N., Bossong, M. G., Boellaard, R., Kloet, R., Schuitemaker, A., Caspers, E., Luurtsema, G., Windhorst, A. D., Cahn, W., Lammertsma, A. A. & Kahn, R. S. (2008). Microglia activation in recent-onset schizophrenia: a quantitative (R)-[11C]PK11195 positron emission tomography study. *Biol. Psychiatry* 64, 820-822.

Van den, B. G. & Arckens, L. (2004). Fluorescent two-dimensional difference gel electrophoresis unveils the potential of gel-based proteomics. *Curr. Opin. Biotechnol.* 15, 38-43.

van Kammen, D. P., lister-Sistilli, C. G., Kelley, M. E., Gurklis, J. A. & Yao, J. K. (1999). Elevated interleukin-6 in schizophrenia. *Psychiatry Res.* 87, 129-136.

van, A. B. & van, S. B. (2011). Drosophila strategies to study psychiatric disorders. *Brain Res. Bull.*

van, O. J. & Kapur, S. (2009). Schizophrenia. *Lancet* 374, 635-645.

van, O. J., Rutten, B. P. & Poulton, R. (2008). Gene-environment interactions in schizophrenia: review of epidemiological findings and future directions. *Schizophr. Bull.* 34, 1066-1082.

Varma, R. & Hoshino, A. Y. (1980). Serum glycoproteins in schizophrenia. *Carbohydr. Res.* 82, 343-351.

Vawter, M. P., Usen, N., Thatcher, L., Ladenheim, B., Zhang, P., VanderPutten, D. M., Conant, K., Herman, M. M., van Kammen, D. P., Sedvall, G., Garver, D. L. & Freed, W. J. (2001). Characterization of human cleaved N-CAM and association with schizophrenia. *Exp. Neurol.* 172, 29-46.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Huson, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L., Skupski, M., Subramanian, G., Thomas, P. D., Zhang, J., Gabor Miklos, G. L., Nelson, C., Broder, S., Clark, A. G., Nadeau, J., McKusick, V. A., Zinder, N., Levine, A. J., Roberts, R. J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., bu-Threideh, J., Beasley, E., Biddick, K., Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di, F., V, Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A. E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T. J., Higgins, M. E., Ji, R. R., Ke, Z., Ketchum, K. A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G. V., Milshina, N., Moore, H. M., Naik, A. K., Narayan, V. A., Neelam, B., Nusskern, D., Rusch, D. B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Wides, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S., Zhao, S., Gilbert, D., Baumhueter, S., Spier, G., Carter, C., Cravchik, A., Woodage, T., Ali, F., An, H., Awe, A., Baldwin, D., Baden, H., Barnstead, M., Barrow, I., Beeson, K., Busam, D., Carver, A., Center, A., Cheng, M. L., Curry, L., Danaher, S., Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostin, D., Houck, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., Koduru, S., Love, A., Mann, F., May, D., McCawley, S., McIntosh, T., McMullen, I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., Qureshi, H., Reardon, M., Rodriguez, R., Rogers, Y. H., Romblad, D., Ruhfel, B., Scott, R., Sitter, C., Smallwood, M., Stewart, E., Strong, R., Suh, E., Thomas, R., Tint, N. N., Tse, S., Vech, C., Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J. F., Guigo, R., Campbell, M. J., Sjolander, K. V., Karlak, B., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T., Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Bafna, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A., Baxendale, J., Blick, L., Caminha, M., Carnes-Stine, J., Caulk, P., Chiang, Y. H., Coyne, M., Dahlke, C., Mays, A., Dombroski, M., Donnelly, M., Ely, D., Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, A., Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J.,

Kagan, L., Kraft, C., Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., Murphy, S., Newman, M., Nguyen, T., Nguyen, N. & Nodell, M. (2001). The sequence of the human genome. ***Science*** **291, 1304-1351.**

Verbanck, P. M., Lotstra, F., Gilles, C., Linkowski, P., Mendlewicz, J. & Vanderhaeghen, J. J. (1984). Reduced cholecystokinin immunoreactivity in the cerebrospinal fluid of patients with psychiatric disorders. ***Life Sci.*** **34, 67-72.**

Vermuyten, K., Lowenthal, A. & Karcher, D. (1990). Detection of neuron specific enolase concentrations in cerebrospinal fluid from patients with neurological disorders by means of a sensitive enzyme immunoassay. ***Clin. Chim. Acta*** **187, 69-78.**

Vernon, A. C., Natesan, S., Modo, M. & Kapur, S. (2011). Effect of chronic antipsychotic treatment on brain structure: a serial magnetic resonance imaging study with ex vivo and postmortem confirmation. ***Biol. Psychiatry*** **69, 936-944.**

Vilner, B. J., John, C. S. & Bowen, W. D. (1995). Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines. ***Cancer Res.*** **55, 408-413.**

von, E. F., Junker, K., Fiedle, W., Wollscheid, V., Durst, M., Claussen, U. & Ernst, G. (2001). Mass spectrometry meets chip technology: a new proteomic tool in cancer research? ***Electrophoresis*** **22, 2898-2902.**

Wahlbeck, K., Ahokas, A., Nikkila, H., Miettinen, K. & Rimon, R. (2000). Cerebrospinal fluid angiotensin-converting enzyme (ACE) correlates with length of illness in schizophrenia. ***Schizophr. Res.*** **41, 335-340.**

Wahlbeck, K., Rimon, R. & Fyhrquist, F. (1993). Elevated angiotensin-converting enzyme (kininase II) in the cerebrospinal fluid of neuroleptic-treated schizophrenic patients. ***Schizophr. Res.*** **9, 77-82.**

Walker, A. M., Peabody, C. A., Ho, T. W. & Warner, M. D. (1992). 50 kD prolactin binding protein in schizophrenics on neuroleptic medication. ***J Psychiatry Neurosci.*** **17, 61-67.**

Wan, C., La, Y., Zhu, H., Yang, Y., Jiang, L., Chen, Y., Feng, G., Li, H., Sang, H., Hao, X., Zhang, G. & He, L. (2007). Abnormal changes of plasma acute phase proteins in schizophrenia and the relation between schizophrenia and haptoglobin (Hp) gene. ***Amino. Acids*** **32, 101-108.**

Wan, C., Yang, Y., Li, H., La, Y., Zhu, H., Jiang, L., Chen, Y., Feng, G. & He, L. (2006). Dysregulation of retinoid transporters expression in body fluids of schizophrenia patients. ***J Proteome. Res.*** **5, 3213-3216.**

Wang, H., Jin, Y., Reddy, M. V., Podolsky, R., Liu, S., Yang, P., Bode, B., Reed, J. C., Steed, R. D., Anderson, S. W., Steed, L., Hopkins, D., Huang, Y. & She, J. X. (2010). Genetically dependent ERBB3 expression modulates antigen presenting cell function and type 1 diabetes risk. ***PLoS. One.*** **5, e11789.**

Wang, S., Zhang, X. & Regnier, F. E. (2002). Quantitative proteomics strategy involving the selection of peptides containing both cysteine and histidine from tryptic digests of cell lysates. ***J Chromatogr. A*** **949, 153-162.**

Watanabe, Y., Someya, T. & Nawa, H. (2010). Cytokine hypothesis of schizophrenia pathogenesis: evidence from human studies and animal models. ***Psychiatry Clin. Neurosci.*** **64, 217-230.**

Watanabe, Y., Usada, N., Minami, H., Morita, T., Tsugane, S., Ishikawa, R., Kohama, K., Tomida, Y. & Hidaka, H. (1993). Calvasculin, as a factor affecting the microfilament assemblies in rat fibroblasts transfected by src gene. ***FEBS Lett.*** **324, 51-55.**

Webster, M. J., Shannon, W. C., Herman, M. M., Hyde, T. M. & Kleinman, J. E. (2001). Synaptophysin and GAP-43 mRNA levels in the hippocampus of subjects with schizophrenia. ***Schizophr. Res.*** **49, 89-98.**

Weickert, C. S., Webster, M. J., Hyde, T. M., Herman, M. M., Bachus, S. E., Bali, G., Weinberger, D. R. & Kleinman, J. E. (2001). Reduced GAP-43 mRNA in dorsolateral prefrontal cortex of patients with schizophrenia. ***Cereb. Cortex*** **11, 136-147.**

Weiler, R., Lassmann, H., Fischer, P., Jellinger, K. & Winkler, H. (1990). A high ratio of chromogranin A to synaptin/synaptophysin is a common feature of brains in Alzheimer and Pick disease. ***FEBS Lett.*** **263, 337-339.**

Weinberger, D. R. (1987). Implications of normal brain development for the pathogenesis of schizophrenia. ***Arch. Gen. Psychiatry*** **44, 660-669.**

Weinberger, D. R. (1996). On the plausibility of "the neurodevelopmental hypothesis" of schizophrenia. ***Neuropsychopharmacology*** **14, 1S-11S.**

Wenk, M. R. (2005). The emerging field of lipidomics. ***Nat. Rev. Drug Discov.*** **4, 594-610.**

Widerlov, E., Lindstrom, L. H., Besev, G., Manberg, P. J., Nemeroff, C. B., Breese, G. R., Kizer, J. S. & Prange, A. J., Jr. (1982). Subnormal CSF levels of neurotensin in a subgroup of schizophrenic patients: normalization after neuroleptic treatment. ***Am J Psychiatry*** **139, 1122-1126.**

Wiener, H. W., Klei, L., Irvin, M. D., Perry, R. T., Aliyu, M. H., Allen, T. B., Bradford, L. D., Calkins, M. E., Devlin, B., Edwards, N., Gur, R. E., Gur, R. C., Kwentus, J., Lyons, P. D., McEvoy, J. P., Nasrallah, H. A., Nimgaonkar, V. L., O'Jile, J., Santos, A. B., Savage, R. M. & Go, R. C. (2009). Linkage analysis of schizophrenia in African-American families. ***Schizophr. Res.*** **109, 70-79.**

Wittke, S., Mischak, H., Walden, M., Kolch, W., Radler, T. & Wiedemann, K. (2005). Discovery of biomarkers in human urine and cerebrospinal fluid by capillary electrophoresis coupled to mass spectrometry: towards new diagnostic and therapeutic approaches. ***Electrophoresis*** **26, 1476-1487.**

Wood, S. J., Yucel, M., Pantelis, C. & Berk, M. (2009). Neurobiology of schizophrenia spectrum disorders: the role of oxidative stress. ***Ann. Acad. Med. Singapore*** **38, 396.**

Woolley, D. W. & Shaw, E. (1954). A biochemichal and pharmacological suggestion about certain mental disorders. ***Proc. Natl. Acad. Sci. U. S. A*** **40, 228-231.**

Xiong, P., Zeng, Y., Zhu, Z., Tan, D., Xu, F., Lu, J., Wan, J. & Ma, M. (2010). Reduced NGF serum levels and abnormal P300 event-related potential in first episode schizophrenia. ***Schizophr. Res.*** **119, 34-39.**

Xu, J., Zhong, N., Wang, H., Elias, J. E., Kim, C. Y., Woldman, I., Pifl, C., Gygi, S. P., Geula, C. & Yankner, B. A. (2005). The Parkinson's disease-associated DJ-1 protein is a transcriptional co-activator that protects against neuronal apoptosis. ***Hum. Mol Genet.*** **14, 1231-1241.**

Yamaguchi, Y. & Pasquale, E. B. (2004). Eph receptors in the adult brain. ***Curr. Opin. Neurobiol.*** **14, 288-296.**

Yang, H., Chen, J., Yang, J., Qiao, S., Zhao, S. & Yu, L. (2007). Cyclophilin A is upregulated in small cell lung cancer and activates ERK1/2 signal. ***Biochem. Biophys. Res. Commun.*** **361, 763-767.**

Yang, S. D., Yu, J. S., Lee, T. T., Yang, C. C., Ni, M. H. & Yang, Y. Y. (1995). Dysfunction of protein kinase FA/GSK-3 alpha in lymphocytes of patients with schizophrenic disorder. ***J Cell Biochem.*** **59, 108-116.**

Yang, Y., Wan, C., Li, H., Zhu, H., La, Y., Xi, Z., Chen, Y., Jiang, L., Feng, G. & He, L. (2006). Altered levels of acute phase proteins in the plasma of patients with schizophrenia. ***Anal. Chem.*** **78, 3571-3576.**

Yin, G. N., Lee, H. W., Cho, J. Y. & Suk, K. (2009). Neuronal pentraxin receptor in cerebrospinal fluid as a potential biomarker for neurodegenerative diseases. ***Brain Res.*** **1265, 158-170.**

Yip, T. T. & Lomas, L. (2002). SELDI ProteinChip array in oncoproteomic research. ***Technol. Cancer Res. Treat.*** **1, 273-280.**

Young-Pearse, T. L., Suth, S., Luth, E. S., Sawa, A. & Selkoe, D. J. (2010). Biochemical and functional interaction of disrupted-in-schizophrenia 1 and amyloid precursor protein regulates neuronal migration during mammalian cortical development. ***J Neurosci.*** **30, 10431-10440.**

Yu, P. H., Bowen, R., Carlson, K., O'Sullivan, K. & Boulton, A. A. (1982). Comparison of biochemical properties of platelet monoamine oxidase in mentally disordered and healthy individuals. ***Psychiatry Res.*** **6, 107-121.**

Zabel, C. & Klose, J. (2004). Influence of Huntington's disease on the human and mouse proteome. ***Int. Rev. Neurobiol.*** **61, 241-283.**

Zhang, H. X., Zhao, J. P., Lv, L. X., Li, W. Q., Xu, L., Ouyang, X., Yuan, Z. Q. & Huang, J. S. (2008). Explorative study on the expression of neuregulin-1 gene in peripheral blood of schizophrenia. ***Neurosci. Lett.*** **438, 1-5.**

Zhang, L., Yang, H., Zhao, H. & Zhao, C. (2010a). Calcium-related signaling pathways contributed to dopamine-induced cortical neuron apoptosis. ***Neurochem. Int.***

Zhang, X. Y., Xiu, M. H., Song, C., Chen, d. C., Wu, G. Y., Haile, C. N., Kosten, T. A. & Kosten, T. R. (2010b). Increased serum S100B in never-medicated and medicated schizophrenic patients. ***J Psychiatr. Res.*** **44, 1236-1240.**

Zheng, J., Koblinski, J. E., Dutson, L. V., Feeney, Y. B. & Clevenger, C. V. (2008). Prolyl isomerase cyclophilin A regulation of Janus-activated kinase 2 and the progression of human breast cancer. ***Cancer Res.*** **68, 7769-7778.**

Zhou, D. & Chen, S. (2001). PNRC2 is a 16 kDa coactivator that interacts with nuclear receptors through an SH3-binding motif. ***Nucleic Acids Res.*** **29, 3939-3948.**

Zhou, Y., Wang, J., Wang, K., Li, S., Song, X., Ye, Y., Wang, L. & Ying, B. (2010). Association analysis between the rs11136000 single nucleotide polymorphism in clusterin gene, rs3851179 single nucleotide polymorphism in clathrin assembly lymphoid myeloid protein gene and the patients with schizophrenia in the Chinese population. ***DNA Cell Biol.*** **29, 745-751.**

Zhu, C., Wang, X., Deinum, J., Huang, Z., Gao, J., Modjtahedi, N., Neagu, M. R., Nilsson, M., Eriksson, P. S., Hagberg, H., Luban, J., Kroemer, G. & Blomgren, K. (2007). Cyclophilin A participates in the nuclear translocation of apoptosis-inducing factor in neurons after cerebral hypoxia-ischemia. ***J Exp. Med.*** **204, 1741-1748.**

Zubenko, G. S., Volicer, L., Direnfeld, L. K., Freeman, M., Langlais, P. J. & Nixon, R. A. (1985). Cerebrospinal fluid levels of angiotensin-converting enzyme in Alzheimer's disease, Parkinson's disease and progressive supranuclear palsy. ***Brain Res.*** **328, 215-221.**

APPENDIXES

Appendix 1

LC/MS/MS Analysis of 2D gel spots: Examining the effects of DISC1 knockdown on the proteome of human neural progenitor cells.

Spot No.	Protein I.D.	Species	Accession No.	MW (Da)	pI	No. Peptides Matched	No. Unique Peptides	Peptide Identity Score	Peptide Identity Threshold	Percentage Coverage	Sequence
RSF/NB A	Dihydropteridine reductase	Human	P09417	25773	6.9	3	2	104.0	55.1	12%	MTDSFTEQADQVTAEVGK
								47.0	52.7		EGGLLTLAGAK
RSF/NB B	Peptidyl-prolyl cis-trans isomerase A	Human	P62937	18001	7.68	10	4	90.9	55.0	32%	VNPTVFFDIAVDGEPLGR
								68.8	53.5		VSFELFADKVPK
								58.1	53.3		EGMNIVEAMER
								64.7	53.2		KITIADCGQLE

Appendix 2

LC/MS/MS Analysis of 2D gel spots: Examining the effects of antipsychotic and antidepressant medication in F344 inbred rats.

2D PAGE gel spots from an F344 rat plasma preparation analysed by LC/MS/MS.

Spot No.	Protein I.D.	Species	Accession No.	MW (Da)	pI	No. Peptides Matched	No. Unique Peptides	Peptide Identity Score	Peptide Identity Threshold	Percentage Coverage	Sequence
699	Fibrinogen alpha chain	Rat	P06399	86632	5.51	44	21	43.9	35.1	29%	AQQIQVLQK
								47.4	35.6		DVRDQLIDMK
								57.5	35.5		EINLKDYEGQQK
								47.9	34.4		ETDWPFCSDEDWNHK
								103.1	34.2		EVVKEVVTSDDGSDCGDGMDLGLTHSFSGR
								70.2	34.2		EVVTSDDGSDCGDGMDLGLTHSFSGR
								56.1	35.8		FGSLTSNFK
								59.8	35.5		FGSLTSNFKEFGSK
								141.7	34.9		GDFANANNFDNTFGQVSEDLR
								60.2	35.1		GDFANANNFDNTFGQVSEDLRR
								45.8	35.7		GDKELLIGNEK
								77.9	35.3		GLIDEANQDFTNR
								65.6	35.6		LVTSKGDKELLIGNEK
								71.0	35.1		MHPELGSFYDSR
								57.2	35.5		MKGLIDEANQDFTNR
								65.0	35.7		MSPVPDLVPGSFK
								23.9	34.2		NIMEYLR
								33.9	35.1		NSLFDFQK
								52.8	35.4		QLEQVIAK
								46.8	35.3		SQLQEGPPEWK
								83.2	34.5		TSDSDIFTDIENPSSHVPEFSSSSK
	Fibrinogen beta chain	Rat	P14480	54201	7.90	29	15	48.5	35.6	37%	ECEEIIR
								73.1	34.5		QDGSVDFGR
								57.3	35.4		KGFGNIATNEDTKK
								47.2	35.4		RKEEPPSLRPAPPPISGGGYR

								57.0	35.1		GFGNIATNEDTK
								54.7	35.6		DNDGWVTTDPR
								69.6	35.7		TENGGWTVIQNR
								45.9	35.3		GFGNIATNEDTKK
								66.3	35.5		GGETSEMYLIQPDTSSKPYR
								49.5	35.4		YCGLPGEYWLGNDKISQLTR
								53.0	35.4		KGGETSEMYLIQPDTSSKPYR
								90.8	35.1		YYWGGLYSWDMSK
								65.2	35.6		IGPTELLIEMEDWK
								104.1	35.6		LYIDETVNDNIPLNLR
								143.3	35.2		DNENVINEYSSILEDQK
	Beta-2-glycoprotein 1	Rat	P26644	33175	8.59	6	4	38.0	35.3	16%	ATVLYQGQR
								43.3	35.6		ITCPPPPIPK
								71.3	35.4		CTEEGKWSPELPVCAR
								79.4	35.6		VCPFAGILENGVVR
	Ig gamma-2B chain C region	Rat	P20761	36474	7.70	3	3	77.5	35.6	17%	CPVPELLGGPSVFIFPPKPK
								80.1	34.8		NTEPVMDSDGSFFMYSK
								39.6	35.5		APFVCSVVHEGLHNHHVEK
	Serotransferrin	Rat	P12346	76346	7.14	1	1	53.1	35.5	2%	TAGWNIPMGLLFSR
694	Fibrinogen alpha chain	Rat	P06399	86632	5.51	41	20	50.8	35.6	29%	DVRDQLIDMK
								63.1	35.5		EINLKDYEGQQK
								54.9	35.3		ETDWPFCSDEDWNHK
								130.2	35.3		EVVKEVVTSDDGSDCGDGMDLGLTHSFSGR
								92.5	35.3		EVVTSDDGSDCGDGMDLGLTHSFSGR
								59.2	35.8		FGSLTSNFK
								128.3	35.7		GDFANANNFDNTFGQVSEDLR
								59.3	35.6		GDFANANNFDNTFGQVSEDLRR
								59.9	35.6		GDKELLIGNEK
								80.2	35.4		GLIDEANQDFTNR

							66.9	35.1		LVTSKGDKELLIGNEK
							41.3	36.0		MELERPGK
							42.2	35.6		MELERPGKDGASR
							70.5	35.3		MHPELGSFYDSR
							69.6	35.7		MSPVPDLVPGSFK
							34.0	34.2		NIMEYLR
							37.7	35.1		NSLFDFQK
							65.2	35.4		QLEQVIAK
							43.1	35.3		SQLQEGPPEWK
							71.0	35.4		TSDSDIFTDIENPSSHVPEFSSSSK
Fibrinogen beta chain	Rat	P14480	54201	7.90	33	19	135.6	35.4	40%	DNENVINEYSSILEDQK
							42.4	35.6		ECEEIIR
							56.5	35.6		ECEEIIRK
							56.6	35.6		GFGNIATNEDTK
							53.9	35.7		GGETSEMYLIQPDTSSKPYR
							75.3	35.6		IGPTELLIEMEDWK
							66.1	35.2		KEEPPSLRPAPPPISGGGYR
							98.9	35.3		KGFGNIATNEDTK
							76.0	35.3		KGFGNIATNEDTKK
							69.5	35.5		KGGETSEMYLIQPDTSSKPYR
							102.5	35.4		KPPDAGGCVHGDGDMGVLCPTGCELR
							124.0	35.2		LYIDETVNDNIPLNLR
							82.5	35.3		QAQVKDNENVINEYSSILEDQK
							62.7	34.5		QDGSVDFGR
							55.8	35.1		QTLLNHERPIK
							57.0	34.9		RKEEPPSLRPAPPPISGGGYR
							51.4	35.6		VYCDMKTENGGWTVIQNR
							49.2	35.7		YCGLPGEYWLGNDK
							65.6	35.7		TENGGWTVIQNR
Beta-2-glycoprotein 1	Rat	P26644	33175	8.59	2	2	73.3	35.6	8%	VCPFAGILENGVVR

								42.2	35.6		ITCPPPPIPK
	Ig gamma-2B chain C region	Rat	P20761	36474	7.70	2	1	62.0	34.8	6%	CPVPELLGGPSVFIFPPKPK
668	Fibrinogen beta chain	Rat	P14480	54201	7.90	56	27	150.0	35.4	56%	DNENVINEYSSILEDQK
								46.1	35.6		ECEEIIR
								58.2	35.6		ECEEIIRK
								57.1	34.9		EDGGGWWYNR
								57.8	35.1		EEPPSLRPAPPPISGGGYR
								58.7	35.1		GFGNIATNEDTK
								53.3	35.7		GGETSEMYLIQPDTSSKPYR
								72.0	35.5		IGPTELLIEMEDWK
								45.8	35.0		KEEPPSLRPAPPPISGGGYR
								98.9	35.3		KGFGNIATNEDTK
								62.5	35.3		KGFGNIATNEDTKK
								76.9	35.4		KGGETSEMYLIQPDTSSKPYR
								94.4	35.4		KPPDAGGCVHGDGDMGVLCPTGCELR
								85.4	35.5		KQCSKEDGGGWWYNR
								44.7	35.1		KYCGLPGEYWLGNDKISQLTR
								113.2	35.2		LYIDETVNDNIPLNLR
								119.6	35.2		QAQVKDNENVINEYSSILEDQK
								55.7	35.5		QCSKEDGGGWWYNR
								61.6	34.5		QDGSVDFGR
								39.4	34.9		QDGSVDFGRK
								58.5	35.0		QTLLNHERPIK
								48.4	34.7		RKEEPPSLRPAPPPISGGGYR
								61.5	35.7		TENGGWTVIQNR
								43.8	35.6		VYCDMKTENGGWTVIQNR
								68.1	35.7		YCGLPGEYWLGNDK
								45.1	35.3		YCGLPGEYWLGNDKISQLTR
								61.2	35.5		YYWGGLYSWDMSK

	Fibrinogen alpha chain	Rat	P06399	86632	5.51	24	15	38.7	34.9	25%	AQQIQVLQK
								47.0	35.6		DVRDQLIDMK
								48.0	35.3		ETDWPFCSDEDWNHK
								84.7	35.3		EVVKEVVTSDDGSDCGDGMDLGLTHSFSGR
								61.9	35.3		EVVTSDDGSDCGDGMDLGLTHSFSGR
								144.9	35.7		GDFANANNFDNTFGQVSEDLR
								58.5	35.6		GDFANANNFDNTFGQVSEDLRR
								80.1	35.4		GLIDEANQDFTNR
								71.3	35.6		MADEAASEAHQEGDTR
								33.0	35.6		MELERPGKDGASR
								60.2	35.3		MHPELGSFYDSR
								64.7	35.6		MSPVPDLVPGSFK
								56.4	35.4		QLEQVIAK
								45.3	35.3		SQLQEGPPEWK
								80.4	35.4		TSDSDIFTDIENPSSHVPEFSSSSK
	Beta-2-glycoprotein 1	Rat	P26644	33175	8.59	5	4	53.7	35.2	11%	ATVLYQGQR
								55.8	35.6		ITCPPPPIPK
								48.2	35.2		KATVLYQGQR
								62.8	35.5		VCPFAGILENGVVR
	Ig gamma-2B chain C region	Rat	P20761	36474	7.70	1	1	52.8	35.5	5%	NTEPVMDSDGSFFMYSK
	Anionic trypsin-2	Rat	P00763	26211	4.77	1	1	52.5	35.6	4%	TLNNDIMLIK
708	Fibrinogen beta chain	Rat	P14480	54201	7.90	49	24	43.7	35.6	55%	DNDGWVTTDPR
								130.8	35.2		DNENVINEYSSILEDQK
								57.2	34.6		DNENVINEYSSILEDQKLYIDETVNDNIPLNLR
								48.7	35.6		ECEEIIR
								45.6	35.6		ECEEIIRK
								57.2	34.6		EDGGGWWYNR
								58.0	35.1		GFGNIATNEDTK

							58.2	35.6		GGETSEMYLIQPDTSSKPYR
							68.5	35.6		IGPTELLIEMEDWK
							62.1	35.6		KEEPPSLRPAPPPISGGGYR
							65.0	35.4		KGFGNIATNEDTKK
							81.9	35.5		KGGETSEMYLIQPDTSSKPYR
							78.8	34.6		KPPDAGGCVHGDGDMGVLCPTGCELR
							103.6	35.6		LYIDETVNDNIPLNLR
							144.4	35.3		NSIAELNSNINSVSETSSVTFQYLTLLK
							66.1	35.4		QAQVKDNENVINEYSSILEDQK
							68.4	34.5		QDGSVDFGR
							44.4	35.6		QTLLNHERPIK
							51.1	35.4		RKEEPPSLRPAPPPISGGGYR
							59.4	35.7		TENGGWTVIQNR
							39.5	35.5		YCGLPGEYWLGNDK
							51.0	35.4		YCGLPGEYWLGNDKISQLTR
							40.7	34.4		YQVSVNK
							77.7	35.2		YYWGGLYSWDMSK
Fibrinogen alpha chain	Rat	P06399	86632	5.51	21	13	43.7	35.1	21%	AQQIQVLQK
							46.2	35.6		DVRDQLIDMK
							80.9	34.3		EVVKEVVTSDDGSDCGDGMDLGLTHSFSGR
							60.4	34.1		EVVTSDDGSDCGDGMDLGLTHSFSGR
							138.5	35.1		GDFANANNFDNTFGQVSEDLR
							62.9	35.2		GDFANANNFDNTFGQVSEDLRR
							53.9	35.7		GDKELLIGNEK
							76.4	35.4		GLIDEANQDFTNR
							62.4	35.2		MHPELGSFYDSR
							64.7	35.7		MSPVPDLVPGSFK
							45.0	35.7		RLEVDIDIK
							45.3	35.3		SQLQEGPPEWK
							78.3	34.7		TSDSDIFTDIENPSSHVPEFSSSSK

	Ig gamma-2B chain C region	Rat	P20761	36474	7.70	7	5	41.7	35.5	24%	APFVCSVVHEGLHNHHVEK
								54.0	35.5		CPTCPTCHK
								54.9	35.5		VVSALPIQHQDWMSGK
								78.2	35.5		CPVPELLGGPSVFIFPPKPK
								73.3	34.9		NTEPVMDSDGSFFMYSK
	Beta-2-glycoprotein 1	Rat	P26644	33175	8.59	1	1	67.4	35.6	5%	VCPFAGILENGVVR
711	Fibrinogen alpha chain	Rat	P06399	86632	5.51	8	5	181.3	32.6	9%	GDFANANNFDNTFGQVSEDLR
								48.5	32.3		GDFANANNFDNTFGQVSEDLRR
								76.3	32.8		GLIDEANQDFTNR
								42.6	33.0		MSPVPDLVPGSFK
								54.4	31.7		TSDSDIFTDIENPSSHVPEFSSSSK
	Fibrinogen beta chain	Rat	P14480	54201	7.90	4	3	94.3	32.4	10%	DNENVINEYSSILEDQK
								43.3	32.9		IGPTELLIEMEDWK
								83.5	32.8		LYIDETVNDNIPLNLR
	Ig gamma-2B chain C region	Rat	P20761	36474	7.70	4	2	71.3	32.8	11%	CPVPELLGGPSVFIFPPKPK
								53.9	31.8		NTEPVMDSDGSFFMYSK
661	Fibrinogen alpha chain	Rat	P06399	86632	5.51	30	19	38.9	35.1	29%	AQQIQVLQK
								46.0	35.5		DVRDQLIDMK
								47.7	35.5		EINLKDYEGQQK
								48.6	34.1		ETDWPFCSDEDWNHK
								91.7	34.0		EVVKEVVTSDDGSDCGDGMDLGLTHSFSGR
								60.1	33.9		EVVTSDDGSDCGDGMDLGLTHSFSGR
								61.8	35.8		FGSLTSNFK
								66.8	35.5		FGSLTSNFKEFGSK
								136.3	34.9		GDFANANNFDNTFGQVSEDLR
								52.2	35.0		GDFANANNFDNTFGQVSEDLRR
								56.4	35.7		GDKELLIGNEK

								98.2	35.3		GLIDEANQDFTNR
								71.1	34.5		MADEAASEAHQEGDTR
								45.6	35.1		MHPELGSFYDSR
								64.8	35.7		MSPVPDLVPGSFK
								33.8	35.1		NSLFDFQK
								57.6	35.4		QLEQVIAK
								43.8	35.3		SQLQEGPPEWK
								58.9	34.4		TSDSDIFTDIENPSSHVPEFSSSSK
	Pyruvate kinase isozymes M1/M2	Rat	P11980	57781	6.63	5	3	74.2	35.5	8%	FGVEQDVDMVFASFIR
								40.1	35.6		LDIDSAPITAR
								71.9	35.7		NTGIICTIGPASR
946	Alpha-1-macroglobulin	Rat	Q63041	2E+05	6.46	8	7	60.8	32.2	5%	DLSSSDLTTASK
								67.2	32.9		KLQDQSNIQR
								42.0	33.0		LADLPGNYITK
								50.3	32.1		LLLQEVR
								72.2	33.1		LQDQSNIQR
								60.9	32.6		QQNSHGGFSSTQDTVVALQALSK
								51.5	32.9		YNILPEAEGEAPFTLK
947	Alpha-1-macroglobulin	Rat	Q63041	2E+05	6.46	15	11	60.8	32.2	8%	DLSSSDLTTASK
								57.6	32.6		KLQDQSNIQR
								54.3	32.6		LADLPGNYITK
								50.2	31.6		LLLQEVR
								75.4	33.0		LQDQSNIQR
								39.2	31.0		NLKPAPVK
								77.5	31.0		NLKPAPVKVYDYYETDEFAIEEYSAPFSSDSEQGNA
								86.7	32.2		TEVNTNHVLIYIEK
								95.7	31.6		VYDYYETDEFAIEEYSAPFSSDSEQGNA
								69.5	33.3		YGAATFTK
								81.6	32.4		YNILPEAEGEAPFTLK
	Tubulin alpha-1C chain	Rat	Q6AYZ1	49905	4.96	2	1	72.1	32.2	3%	AVFVDLEPTVIDEVR

943	Alpha-1-macroglobulin	Rat	Q63041	2E+05	6.46	7	7	67.2	32.2	6%	DLSSSDLTTASK
								56.3	32.8		LADLPGNYITK
								43.4	31.7		LLLQEVR
								58.2	33.1		LQDQSNIQR
								80.2	32.3		QQNSHGGFSSTQDTVVALQALSK
								68.6	32.3		TEVNTNHVLIYIEK
								86.5	32.6		YNILPEAEGEAPFTLK
1669	Fibrinogen beta chain	Rat	P14480	54201	7.90	56	24	135.2	32.0	54%	DNENVINEYSSILEDQK
								48.6	33.0		ECEEIIR
								40.1	33.0		ECEEIIRK
								57.2	31.4		EDGGGWWYNR
								77.3	32.4		GFGNIATNEDTK
								54.6	32.2		GGETSEMYLIQPDTSSKPYR
								74.2	32.1		HGTDDGVVWMNWK
								76.2	32.7		IGPTELLIEMEDWK
								50.1	32.9		KEEPPSLRPAPPPISGGGYR
								75.7	32.5		KGFGNIATNEDTK
								77.6	32.5		KGFGNIATNEDTKK
								71.7	32.1		KGGETSEMYLIQPDTSSKPYR
								44.0	31.4		KPPDAGGCVHGDGDMGVLCPTGCELR
								66.9	31.9		KQCSKEDGGGWWYNR
								95.9	32.7		LYIDETVNDNIPLNLR
								107.2	31.9		QAQVKDNENVINEYSSILEDQK
								89.4	32.0		QDGSVDFGR
								72.2	32.6		QTLLNHERPIK
								57.1	32.7		RKEEPPSLRPAPPPISGGGYR
								62.2	33.0		TENGGWTVIQNR
								48.6	31.3		TMTIHNGMFFSTYDRDNDGWVTTDPR
								50.9	32.1		YCGLPGEYWLGNDK
								38.8	32.2		YCGLPGEYWLGNDKISQLTR
								71.8	31.8		YYWGGLYSWDMSK

763	Fibrinogen gamma chain	Rat	P02680	50600	5.62	36	19	37.2	32.0	59%	AIQVYYNPDQPPKPGMIEGATQK
								69.5	31.9		ATQQFLVYCEIDGSGNGWTVLQK
								54.0	31.6		CHAGHLNGVYYQGGTYSK
								64.4	31.6		DNCCILDER
								72.8	31.7		EGFGHLSPTGTTEFWLGNEK
								41.2	32.5		ESGLYFIRPLK
								102.1	30.3		FGSYCPTTCGISDFLNSYQTDVDTDLQTLENILQR
								63.6	31.9		IHDTTGKDCQDIANK
								56.7	33.3		KMVEEILK
								51.1	31.8		LDGSVDFK
								70.6	32.3		LSIGDGQQHHMGGSK
								104.8	32.4		MVEEILKYEALLLTHESSIR
								52.8	32.0		SSTPNGYDNGIIWATWK
								61.6	32.3		TSTADYAMFR
								79.0	31.7		VAQLEAQCQEPCK
								59.8	32.0		VAQLEAQCQEPCKDSVR
								85.7	32.4		VGPESDKYR
								70.6	32.9		YEALLLTHESSIR
								61.1	32.8		YLQDIYTSNK
	Complement C3	Rat	P01026	2E+05	6.12	2	1	76.6	32.5	2%	ILLQGTPVAQMAEDAVDGER
	Fibrinogen beta chain	Rat	P14480	54201	7.90	2	1	43.1	32.6	3%	IGPTELLIEMEDWK
985	Haptoglobin	Rat	P06866	38539	6.10	14	10	64.7	32.8	25%	ATDLKDWVQETMAK
								51.8	32.8		ATDLKDWVQETMAKN
								74.7	32.7		DIAPTLTLYVGK
								33.4	32.5		DWVQETMAKN
								63.6	32.3		GAVSPVGVQPILNK
								48.5	33.1		IIGGSMDAK
								62.4	32.2		MGYVSGWGR
								53.2	33.0		NQLVEIEK
								43.7	32.5		SVVDIGLIK
								54.4	32.8		YVMLPVADQEK

	Anionic trypsin-2	Rat	P00763	26211	4.77	1	1	51.1	32.9	4%	TLNNDIMLIK
1681	Plasminogen	Rat	Q01177	90477	6.79	19	13	57.7	32.0	16%	CEGETDFICR
								52.9	32.7		CTTPPPPPGPTYQCLK
								40.8	33.1		DVILFEKR
								36.4	32.3		EAQLPVIENK
								83.7	32.4		EQQCVIMAENSK
								45.2	32.9		IFTPQTNPR
								89.6	32.5		ILGSDVQQIAVTK
								55.8	32.5		LKEAQLPVIENK
								35.3	31.7		NLEENYCR
								79.6	33.1		QLAAGSIADCLAK
								64.7	32.7		TPENFPCK
								43.5	31.7		YSPSTHPSEGLEENYCR
								33.0	32.4		YVNWIER
	Serotransferrin	Rat	P12346	76346	7.14	2	1	74.5	32.5	2%	EGVCPEGSIDSAPVK
1126	Carbonic anhydrase 2	Rat	P27139	29096	6.89	19	2	31.7	33.1	9%	EFPIANGDR
								34.7	32.8		EPITVSSEQMSHFR
	Ig kappa chain C region, A allele	Rat	P01836	11725	4.99	6	2	30.9	32.6	25%	DGVLDSVTDQDSK
								51.3	32.6		DSTYSMSSTLSLTK
	Ig lambda-2 chain C region	Rat	P20767	11311	5.76	4	2	41.8	32.6	32%	ANGAPISQGVDTANPTK
								36.6	32.8		NSFTCQVTHEGNTVEK
	Carbonic anhydrase 1	Rat	B0BNN3	28282	6.86	3	1	43.1	32.2	4%	YSSAAEAISK
862	Apolipoprotein A-IV	Rat	P02651	44429	5.12	81	16	42.8	33.0	51%	VSTNIDQLQK
								52.2	32.5		LAPLAEGVQEK
								33.0	32.4		VSQMFGDNVQK
								32.3	32.9		QLDQQVEVFR
								37.9	31.1		ATIDQNLEDLR

								40.7	32.3		NLAPLVEDVQSK
								35.7	32.6		EKVSSFMSTLQK
								47.6	31.1		RAVEPLGDKFNMALVQQMEK
								31.5	32.9		LGNINTYADDLQNK
								62.8	32.5		FRQQLGSDSGDVESHLSFLEK
								47.4	32.2		TDVTQQLNTLFQDK
								41.6	32.8		QQLGSDSGDVESHLSFLEK
								42.4	31.2		TDVTQQLNTLFQDKLGNINTYADDLQNK
								38.6	31.7		AVEPLGDKFNMALVQQMEK
								49.2	31.3		KGSPDQPLALPLPEQVQEQVQEQVQPKPLES
								34.8	32.4		MQTTIQDNVENLQSSMVPFANELK
	ATP synthase subunit beta, mitochondrial	Rat	P10719	56318	5.19	6	2	55.2	32.8	5%	AHGGYSVFAGVGER
								40.9	32.8		VALVYGQMNEPPGAR
837	Complement C3	Rat	P01026	2E+05	6.12	31	3	47.3	32.8	2%	AAVFNHFISDGVK
								44.7	32.8		ILLQGTPVAQMAEDAVDGER
								44.9	31.3		ILLQGTPVAQMAEDAVDGERLK
1679	Alpha-1-antiproteinase	Rat	P17475	46107	5.7	33	8	47.2	32.8	27%	ISSNLADFAFSLYR
								46.0	32.6		FDHPFIFMIVESETQSPLFVGK
								41.7	32.5		KISSNLADFAFSLYR
								57.3	32.5		VFNNDADLSGITEDAPLK
								30.6	32.3		FLEEVKNNYHSEAFSVNFADSEEAK
								32.4	31.8		GTEAAGATVVEAVPMSLPPQVK
								40.5	32.8		NNYHSEAFSVNFADSEEAK
								36.3	32.6		MQHLEQTLTK
	Angiotensinogen	Rat	P01015	51949	5.37	5	3	40.5	32.6	11%	GSYNLQDLLAQAK
								52.0	32.4		SLDLSTDPVLAAQK
								42.4	32.5		AAQVAMIANFMGFR
	Fibrinogen gamma chain	Rat	P02680	50600	5.62	13	2	43.7	32.7	7%	TSTADYAMFR
								31.8	32.6		EGFGHLSPTGTTEFWLGNEK
	Vitamin D-binding protein	Rat	P04276	53509	5.65	9	1	52.3	32.9	4%	DLCGQSATQAMDQYTFELSR

1127	Carbonic anhydrase 2	Rat	P27139	29096	6.89	17	3	51.2	32.8	20%	EPITVSSEQMSHFR
								47.4	31.3		AVQHPDGLAVLGIFLK
								32.4	31.7		SIVNNGHSFNVEFDDSQDFAVLK
	Carbonic anhydrase 1	Rat	B0BNN3	28282	6.86	6	2	34.4	32.2	11%	VGPANPNLQK
								39.2	32.5		HDSSLKPVSVSYNPATAK
	Ig kappa chain C region, A allele	Rat	P01836	11725	4.99	4	2	45.5	32.8	25%	DGVLDSVTDQDSK
								37.6	32.7		DSTYSMSSTLSLTK
	Ig lambda-2 chain C region	Rat	P20767	11311	5.76	2	2	52.5	32.7	32%	ANGAPISQGVDTANPTK
								43.4	32.8		NSFTCQVTHEGNTVEK
240	Complement C3	Rat	P01026	2E+05	6.12	9	3	39.0	32.8	3%	ILLQGTPVAQMAEDAVDGER
								35.1	32.8		VYSYYNLEESCTR
								35.8	32.9		DICEGQVNSLPGSINK

LC/MS/MS analysis of 2D PAGE gel spots from an F344 rat striatum preparation.

Spot No.	Protein I.D.	Species	Accession No.	MW (Da)	pI	No. Peptides Matched	No. Unique Peptides	Peptide Identity Score	Peptide Identity Threshold	Percentage Coverage	Sequence
1143	Dihydropteridine reductase	Rat	P11348	25536	7.67	20	12	69.6	32.7	66%	AALDGTPGMIGYGMAK
								59.8	33.0		EGGLLTLAGAK
								87.0	32.4		GAVHQLCQSLAGK
								70.9	32.7		LLGDQKVDAILCVAGGWAGGNAK
								130.2	32.8		MTDSFTEQADQVTAEVGK
								36.5	32.0		NCDLMWK
								95.3	32.8		NSGMPSGAAAIAVLPVTLDTPMNR
								51.6	32.5		NSGMPSGAAAIAVLPVTLDTPMNRK
								51.2	32.5		QSIWTSTISSHLATK
								65.8	32.6		RPNSGSLIQVVTTDGKTELTPAYF
								38.0	32.9		RVLVYGGR
								60.2	32.8		VDAILCVAGGWAGGNAK
	Triosephosphate isomerase	Rat	P48500	26832	6.89	1	1	59.6	32.4	5%	IIYGGSVTGATCK
1163	Triosephosphate isomerase	Rat	P48500	26832	6.89	36	15	68.5	32.8	77%	AIADNVKDWCK
								53.9	32.7		CLGELICTLNAAK
								61.6	31.0		CLGELICTLNAAKLPADTEVVCAPPTAYIDFAR
								68.3	32.5		CNVSEGVAQCTR
								44.9	31.1		ELASQPDVDGFLVGGASLKPEFVDIINAKQ
								76.2	32.5		HIFGESDELIGQK
								74.6	32.3		IAVAAQNCYK

								81.6	32.2		IIYGGSVTGATCK
								54.1	31.9		KFFVGGNWK
								74.8	32.5		LPADTEVVCAPPTAYIDFAR
								139.4	31.8		VNHALSEGLGVIACIGEK
								75.8	31.8		VNHALSEGLGVIACIGEKLDER
											VNHALSEGLGVIACIGEKLDEREA
								76.3	31.4		GITEK
								84.7	32.5		VTNGAFTGEISPGMIK
								82.5	32.1		VVLAYEPVWAIGTGK
877	Aspartate aminotransferase, cytoplasmic	Rat	P13221	46400	6.73	11	8	89.8	32.7	26%	APPSFFAQVPQAPPVLVFK
								51.8	32.9		IANDHSLNHEYLPILGLAEFR
								59.3	33.0		IGADFLGR
								71.0	32.6		NLDYVATSINEAVTK
								98.0	32.8		NLDYVATSINEAVTKFQ
								76.7	32.9		SCASQLVLGDNSPALR
								103.9	32.5		TDDSQPWVLPVVR
								82.2	32.6		VGGVQSLGGTGALR
	Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	Rat	Q68FX0	42327	8.89	7	4	70.1	32.4	19%	DMGGYSTTTDFIK
								64.1	32.9		LGDGLFLQCCEEVAELYPK
								111.8	33.0		NIANPTAMLLSASNMLR
											VEGAFPVTMLPGDGVGPELMHA
								75.5	32.8		VK
	Fructose-bisphosphate aldolase A	Rat	P05065	39327	8.31	4	3	46.6	32.8	11%	ALANSLACQGK
								65.7	32.9		FSNEEIAMATVTALR
								76.5	32.9		GILAADESTGSIAK
	Triosephosphate isomerase	Rat	P48500	26832	6.89	2	2	60.0	32.8	11%	VVLAYEPVWAIGTGK
								86.2	32.4		IIYGGSVTGATCK

1113	Carbonic anhydrase 2	Rat	P27139	29096	6.89	11	7	52.0	32.9	33%	SHHWGYSK
								48.7	32.0		QSPVDIDTGTAQHDPSLQPLLICY DK
								56.9	32.1		LNFNSEGEAEELMVDNWRPAQP LK
								48.0	32.3		KLNFNSEGEAEELMVDNWRPAQ PLK
								41.1	32.0		ITEALHSIK
								50.7	32.3		IGPASQGLQK
								63.8	32.2		EGPLSGSYR
	Phosphoglycerate mutase 1	Rat	P25113	28814	6.67	3	3	31.2	31.8	22%	SYDVPPPPMEPDHPFYSNISK
								57.4	32.4		YADLTEDQLPSCESLKDTIAR
								44.2	32.7		ALPFWNEEIVPQIK
445	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	Rat	Q920L2	71570	6.75	35	21	105.8	32.9	55%	AAFGLSEAGFNTACLTK
								39.3	32.7		ACALSIAESCRPGDKVPPIK
								47.3	32.4		AGLPCQDLEFVQFHPTGIYGAGCL ITEGCR
								52.6	32.6		AKNTIIATGGYGR
								98.2	32.8		ANAGEESVMNLDKLR
								62.6	32.4		GCGPEKDHVYLQLHHLPPEQLAT R
								51.1	32.1		GSDWLGDQDAIHYMTEQAPASV VELENYGMPFSR
								96.9	32.5		GVIALCIEDGSIHR
								64.6	32.9		HTLSYVDTK
								86.5	32.7		HVNGQDQIVPGLYACGEAACASV HGANR

								49.6	33.0		IDEYDYSKPIEGQQK
								75.6	32.7		KHTLSYVDTK
								35.5	32.6		KPFAEHWR
								107.6	32.5		LGANSLLDLVVFGR
								41.9	32.6		SMQSHAAVFR
								74.4	32.5		TGHSLLHTLYGR
								67.1	32.5		TYFSCTSAHTSTGDGTAMVTR
											VSDAISTQYPVVDHEFDAVVVGA
								62.5	32.6		GGAGLR
								65.1	32.6		VSQLYGDLQHLK
											VTLDYRPVIDKTLNEADCATVPPAI
								38.2	32.2		R
								39.9	32.0		WHFYDTVK
	Dihydropyrimidinase-related protein 2	Rat	P47942	62239	5.95	2	2	36.7	32.2	5%	QIGENLIVPGGVK
								66.8	32.5		IVLEDGTLHVTEGSGR
1159	Triosephosphate isomerase	Rat	P48500	26832	6.89	26	16	42.5	32.8	80%	AIADNVKDWCK
								52.0	32.9		CLGELICTLNAAK
											CLGELICTLNAAKLPADTEVVCAPP
								88.8	32.1		TAYIDFAR
								73.5	32.3		CNVSEGVAQCTR
								75.5	32.6		DLGATWVVLGHSER
											ELASQPDVDGFLVGGASLKPEFVD
								56.0	32.4		IINAK
								71.6	32.7		HIFGESDELIGQK
								55.9	32.4		IAVAAQNCYK
								81.6	32.4		IIYGGSVTGATCK
								58.1	32.1		KFFVGGNWK
								104.8	32.8		LPADTEVVCAPPTAYIDFAR
								75.1	32.9		RHIFGESDELIGQK
								111.8	32.7		VNHALSEGLGVIACIGEK
								73.7	32.8		VNHALSEGLGVIACIGEKLDER
								72.6	32.8		VTNGAFTGEISPGMIK
								65.9	32.8		VVLAYEPVWAIGTGK

595	Pyruvate kinase isozymes M1/M2	Rat	P11980	57781	6.63	92	43	48.2	31.1	74%	APIIAVTR
								49.0	31.4		MQHLIAR
								59.6	32.5		IISKIENHEGVR
								42.7	33.0		VNLAMNVGK
								56.1	33.2		GSGTAEVELK
								38.8	33.2		LLFEELAR
								41.0	33.1		GDYPLEAVR
								39.9	33.0		EAEAAVFHR
								44.1	32.8		TGLIKGSGTAEVELKK
								67.4	33.0		GSGTAEVELKK
								46.8	32.7		LNFSHGTHEYHAETIKNVR
								56.0	32.5		GDLGIEIPAEK
								65.2	32.7		LDIDSAPITAR
								58.3	32.2		ITLDNAYMEK
								65.5	32.6		SVEMLKEMIK
								78.9	32.6		LNFSHGTHEYHAETIK
								100.9	32.6		GADYLVTEVENGGSLGSK
								64.2	32.8		NTGIICTIGPASR
								70.5	32.7		EKGADYLVTEVENGGSLGSK
								57.6	32.7		EKGADYLVTEVENGGSLGSKK
								92.1	32.8		IYVDDGLISLQVK
								75.7	32.4		CDENILWLDYK
								78.2	32.8		CLAAALIVLTESGR
								49.9	32.7		APIIAVTRNPQTAR
								87.5	32.8		DAVLDAWAEDVDLR
								81.3	32.6		KGVNLPGAAVDLPAVSEKDIQDLK
								113.1	32.7		IYVDDGLISLQVKEK
											AEGSDVANAVLDGADCIMLSGET
								99.7	32.1		AKGDYPLEAVR
								72.8	32.7		GADYLVTEVENGGSLGSKK
								92.6	32.7		RFDEILEASDGIMVAR
								62.5	32.5		GDLGIEIPAEKVFLAQK
								111.0	32.8		FGVEQDVDMVFASFIR

								94.0	32.7		FGVEQDVDMVFASFIRK
								76.7	32.8		AGKPVICATQMLESMIK
								59.5	32.4		AATESFASDPILYRPVAVALDTKGP EIR
								122.3	32.5		ASSQSTDPLEAMAMGSVEASYK
								44.2	32.2		GIFPVLCKDAVLDAWAEDVDLRV NLAMNVGK
								67.0	32.7		GVNLPGAAVDLPAVSEKDIQDLK
								53.9	32.6		AATESFASDPILYRPVAVALDTK
								132.4	32.6		AEGSDVANAVLDGADCIMLSGET AK
								97.0	32.8		GIFPVLCKDAVLDAWAEDVDLR
								30.8	32.8		DAVLDAWAEDVDLRVNLAMNV GK
								60.3	32.8		ITLDNAYMEKCDENILWLDYK
1228	Protein DJ-1	Rat	O88767	19961	6.32	52	19	39.5	30.4	90%	RALVILAK
								59.0	33.0		EILKEQENR
								67.3	32.7		DKMMNGSHYSYSESR
								73.6	32.7		VEKDGLILTSR
								69.8	32.8		GAEEMETVIPVDIMRR
								80.1	32.4		AGIKVTVAGLAGKDPVQCSR
								73.9	32.7		MMNGSHYSYSESR
								52.0	32.6		DVVICPDTSLEEAK
								120.7	32.1		KGLIAAICAGPTALLAHEVGFGCK
								71.8	32.7		VTVAGLAGKDPVQCSR
								35.0	32.8		GAEEMETVIPVDIMR
								98.0	31.9		TQGPYDVVVLPGGNLGAQNLSES ALVK
								56.6	30.3		DVVICPDTSLEEAKTQGPYDVVVL PGGNLGAQNLSESALVK
								140.3	32.3		GPGTSFEFALAIVEALSGK
								48.0	31.0		VEKDGLILTSRGPGTSFEFALAIVE ALSGKDMANQVK

										VEKDGLILTSRGPGTSFEFALAIVE
							51.0	31.3		ALSGK
							101.4	32.1		GLIAAICAGPTALLAHEVGFGCK
										DGLILTSRGPGTSFEFALAIVEALSG
							42.5	31.1		KDMANQVK
										GPGTSFEFALAIVEALSGKDMAN
							92.6	32.1		QVK
	Pyruvate kinase isozymes M1/M2	Rat	P11980	57781	6.63	13	8	78.8	32.4	22%	IYVDDGLISLQVK
							79.7	32.5		CLAAALIVLTESGR
							68.0	32.4		GIFPVLCKDAVLDAWAEDVDLR
							105.8	32.2		IYVDDGLISLQVKEK
							89.6	32.6		RFDEILEASDGIMVAR
							125.2	32.7		FGVEQDVDMVFASFIR
							74.5	32.5		FGVEQDVDMVFASFIRK
										AEGSDVANAVLDGADCIMLSGET
							45.5	32.0		AKGDYPLEAVR
	Glutathione S-transferase P	Rat	P04906	23424	6.89	3	3	66.5	32.6	25%	FEDGDLTLYQSNAILR
							106.0	32.9		EAALVDMVNDGVEDLR
							63.3	32.1		ALPGHLKPFETLLSQNQGGK
	Acyl-protein thioesterase 1	Rat	P70470	24692	6.05	3	2	41.3	32.2	12%	LAGVTALSCWLPLR
							79.0	32.6		ASFSQGPINSANR
968	Glyceraldehyde-3-phosphate dehydrogenase	Rat	P04797	35805	8.14	49	11	49.2	33.0	58%	VGVNGFGR
							70.4	32.7		VVDLMAYMASKE
							51.2	32.6		GAAQNIIPASTGAAK
										VIISAPSADAPMFVMGVNHEKYD
							58.6	32.6		NSLK
							78.1	32.8		LTGMAFRVPTPNVSVVDLTCR
							82.7	32.8		VPTPNVSVVDLTCR
							114.2	32.5		VIHDNFGIVEGLMTTVHAITATQK

								67.8	32.9		LISWYDNEYGYSNR
								87.4	32.8		IVSNASCTTNCLAPLAK
											AAFSCDKVDIVAINDPFIDLNYMV
								44.3	31.6		YMFQYDSTHGK
								133.3	32.6		WGDAGAEYVVESTGVFTTMEK
	Pyruvate kinase isozymes M1/M2	Rat	P11980	57781	6.63	8	4	70.6	32.7	13%	CLAAALIVLTESGR
								70.6	32.8		FDEILEASDGIMVAR
								75.4	32.8		FGVEQDVDMVFASFIR
								96.3	32.8		ASSQSTDPLEAMAMGSVEASYK
	Protein DJ-1	Rat	O88767	19961	6.32	6	3	57.8	32.5	26%	GLIAAICAGPTALLAHEVGFGCK
								102.9	32.6		GPGTSFEFALAIVEALSGK
											GPGTSFEFALAIVEALSGKDMAN
								84.5	32.5		QVK
	Malate dehydrogenase, mitochondrial	Rat	P04636	35661	8.93	4	1	95.5	31.9	6%	VAVLGASGGIGQPLSLLLK
1039	Voltage-dependent anion-selective channel protein 1	Rat	Q9Z2L0	30737	8.62	18	11	70.6	32.9	58%	LTLSALLDGK
								61.2	32.4		LTLSALLDGKNVNAGGHK
								76.8	32.6		VTQSNFAVGYK
								69.7	32.8		KLETAVNLAWTAGNSNTR
								53.3	32.0		GYGFGLIKLDLK
								59.0	32.7		YQVDPDACFSAK
								84.5	32.7		LTFDSSFSPNTGKK
								61.8	32.7		SENGLEFTSSGSANTETTK
								113.2	32.2		VNNSSLIGLGYTQTLKPGIK
								122.1	32.8		WNTDNTLGTEITVEDQLAR
								87.2	32.7		GALVLGYEGWLAGYQMNFETSK
	Voltage-dependent anion-selective channel protein 2	Rat	P81155	31726	7.44	14	10	37.0	32.1	48%	SNFAVGYR
								61.5	33.0		LTLSALVDGK

								62.3	32.6		LTFDTTFSPNTGK
								67.2	32.7		YQLDPTASISAK
								59.7	32.7		LTFDTTFSPNTGKK
								59.1	32.5		DIFNKGFGFGLVK
								33.4	32.8		AECCVPVCQRPICIPPPYADLGK
								106.8	32.6		SCSGVEFSTSGSSNTDTGK
								69.1	32.4		VNNSSLIGVGYTQTLRPGVK
								139.8	32.7		WNTDNTLGTEIAIEDQICQGLK
	Glyceraldehyde-3-phosphate dehydrogenase	Rat	P04797	35805	8.14	7	4	70.1	32.7	29%	VVDLMAYMASKE
								83.5	32.1		VIHDNFGIVEGLMTTVHAITATQK
								121.0	32.6		WGDAGAEYVVESTGVFTTMEK
											GILGYTEDQVVSCDFNSNSHSSTF
								44.7	31.9		DAGAGIALNDNFVK
	V-type proton ATPase subunit E 1	Rat	Q6PCU2	26112	8.44	3	3	89.2	32.6	21%	ARDDLITDLLNEAK
								106.7	32.0		YQVLLDGLVLQGLYQLLEPR
								62.7	32.5		LDLIAQQMMPEVR
729	ATP synthase subunit alpha, mitochondrial	Rat	P15999	59717	9.22	58	32	38.0	33.5	62%	STVAQLVK
								42.4	31.3		RVGLKAPGIIPR
								39.2	32.6		LTDADAMK
								60.3	33.1		QMSLLLR
								37.5	32.9		LELAQYR
								41.6	32.8		VLSIGDGIAR
								32.7	32.9		RSTVAQLVK
								48.1	32.8		RLTDADAMK
								48.5	32.0		AVDSLVPIGR
								43.9	32.5		QVAGTMKLELAQYR
								56.1	31.9		VGLKAPGIIPR
								56.9	32.6		VVDALGNAIDGKGPVGSK
								85.3	32.6		DNGKHALIIYDDLSK

								71.0	32.7		VVDALGNAIDGK
								91.3	32.3		FESAFLSHVVSQHQSLLGNIR
								53.3	32.8		ELIIGDRQTGK
								69.2	32.7		HALIIYDDLSK
								83.7	32.8		TSIAIDTIINQK
								77.4	31.6		ITKFESAFLSHVVSQHQSLLGNIR
								81.1	32.5		TGTAEMSSILEER
								80.9	31.8		GIRPAINVGLSVSR
								80.3	32.6		ILGADTSVDLEETGR
								54.8	32.4		LKEIVTNFLAGFEP
								92.2	32.6		TGAIVDVPVGDELLGR
								96.0	32.9		GMSLNLEPDNVGVVVFGNDK
								43.7	31.9		ILGADTSVDLEETGRVLSIGDGIAR
								61.6	32.1		GMSLNLEPDNVGVVVFGNDKLIK
											TGAIVDVPVGDELLGRVVDALGN
								35.9	31.3		AIDGKGPVGSK
								138.1	32.6		QGQYSPMAIEEQVAVIYAGVR
								130.4	32.7		EVAAFAQFGSDLDAATQQLLSR
								38.3	32.0		ISEQSDAKLKEIVTNFLAGFEP
											MNDSFGGGSLTALPVIETQAGDV
								47.0	29.9		SAYIPTNVISITDGQIFLETELFYK
838	Glutamine synthetase	Rat	P09606	42240	6.64	28	6	44.5	32.9	24%	ATSASSHLNK
								37.2	32.8		CIEEAIDKLSKR
								75.9	32.3		RLTGFHETSNINDFSAGVANR
								47.2	32.9		TCLLNETGDEPFQYK
								38.5	32.3		IQLMYIWVDGTGEGLR
								48.4	32.2		LTGFHETSNINDFSAGVANR
	Phosphoglycerate kinase 1	Rat	P16617	44510	8.02	22	4	61.0	32.6	18%	LGDVYVNDAFGTAHR
								54.6	32.8		GCITIIGGGDTATCCAK
								61.9	32.7		DCVGSEVENACANPAAGTVILLEN LR
								37.1	32.4		ITLPVDFVTADKFDENAK

	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	Rat	P26284	43227	8.49	4	2	34.0	33.3	5%	AAASTDYYK
								43.6	32.9		VDGMDILCVR
	Creatine kinase U-type, mitochondrial	Mouse	P30275	46974	8.39	7	1	31.5	32.9	4%	LGYILTCPSNLGTGLR
963	Glyceraldehyde-3-phosphate dehydrogenase	Rat	P04797	35805	8.14	29	5	51.0	32.9	23%	VVDLMAYMASKE
								56.4	32.5		VPTPNVSVVDLTCR
								34.4	32.9		LISWYDNEYGYSNR
								51.6	32.7		WGDAGAEYVVESTGVFTTMEK
								63.3	32.4		GAAQNIIPASTGAAK
	Malate dehydrogenase, mitochondrial	Rat	P04636	35661	8.93	6	2	30.9	32.8	8%	VNVPVIGGHAGK
								35.3	32.8		VDFPQDQLATLTGR
	NAD-dependent deacetylase sirtuin-2	Rat	Q5RJQ4	39294	6.67	3	2	40.3	32.6	10%	CYTQNIDTLER
								54.2	32.8		EHANIDAQSGSQASNPSATVSPR
908	Fructose-bisphosphate aldolase C	Rat	P09117	39259	6.67	41	10	40.5	31.5	36%	PHSYPALSAEQKK
								33.7	33.0		ALQASALSAWR
								32.0	32.9		IVAPGKGILAADESVGSMAK
								49.6	32.7		GILAADESVGSMAK
								31.6	32.0		YEGSGDGGAAAQSLYVANHAY
								46.9	31.9		ISDRTPSALAILENANVLAR
								36.3	32.7		DNAGAATEEFIKR
								42.1	31.9		TPSALAILENANVLAR

								45.1	31.2		TVPPAVPGVTFLSGGQSEEEASLN LNAINR
								39.5	31.1		RTVPPAVPGVTFLSGGQSEEEASL NLNAINR
961	Glyceraldehyde-3-phosphate dehydrogenase	Rat	P04797	35805	8.14	33	7	46.3	32.7	33%	VVDLMAYMASKE
								42.2	32.8		LEKPAKYDDIKK
								51.4	32.7		VPTPNVSVVDLTCR
								28.3	31.5		VIPELNGK
								32.3	32.9		IVSNASCTTNCLAPLAK
								62.8	32.7		WGDAGAEYVVESTGVFTTMEK
								61.9	32.4		GAAQNIIPASTGAAK
	NAD-dependent deacetylase sirtuin-2	Rat	Q5RJQ4	39294	6.67	7	5	44.0	32.5	17%	SPSTGLYANLEK
								52.4	32.9		LLDELTLEGVTR
								36.7	32.5		CYTQNIDTLER
								64.0	31.7		REHANIDAQSGSQASNPSATVSP R
								54.3	32.6		EHANIDAQSGSQASNPSATVSPR
1142	Ubiquitin carboxyl-terminal hydrolase isozyme L1	Rat	Q00981	24822	5.14	39	8	23.0	32.8	55%	MQLKPMEINPEMLNK
								31.9	32.6		EFTEREQGEVR
								39.0	32.4		QIEELKGQEVSPK
								73.1	31.9		KQIEELKGQEVSPK
								33.7	32.2		QFLSETEKLSPEDR
								34.1	32.5		CFEKNEAIQAAHDSVAQEGQCR
								58.3	32.7		NEAIQAAHDSVAQEGQCR
								53.4	32.3		MPFPVNHGASSEDSLLQDAAK
	Rho GDP-dissociation inhibitor 1	Rat	Q5XI73	23393	5.12	7	2	44.0	32.8	24%	SIQEIQELDKDDESLR

								47.1	32.3		AEQEPTAEQLAQIAAENEEDEHSVNYKPPAQK
	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	Rat	P19234	27361	6.23	3	2	46.4	32.8	10%	DSDSILETLQR
								39.8	32.7		AAAVLPVLDLAQR
1214	Glutathione S-transferase P	Rat	P04906	23424	6.89	15	5	35.4	49.4	30%	PPYTIVYFPVR
								41.0	49.7		DQKEAALVDMVNDGVEDLR
								34.5	49.6		YGTLIYTNYENGKDDYVK
								60.4	49.6		EAALVDMVNDGVEDLR
								33.5	49.7		FEDGDLTLYQSNAILR
642	V-type proton ATPase subunit B, brain isoform	Rat	P62815	56515	5.57	61	11	46.4	32.9	34%	RGFPGYMYTDLATIYER
								49.8	32.7		AVVQVFEGTSGIDAK
								48.0	31.6		QIYPPINVLPSLSR
								40.5	32.2		IYPEEMIQTGISAIDGMNSIAR
								32.5	32.3		TVYETLDIGWQLLR
								46.8	32.2		YAEIVHLTLPDGTKR
								40.8	32.1		DHADVSNQLYACYAIGKDVQAMK
								44.6	32.9		GFPGYMYTDLATIYER
								38.2	31.9		GIVNGAAPELPVPTGGPMAGAR
								50.0	31.9		IPIFSAAGLPHNEIAAQICR
								29.7	32.9		SGQVLEVSGSK
	Tubulin beta-2C chain	Rat	Q6P9T8	49769	4.79	19	2	32.0	32.7	7%	EIVHLQAGQCGNQIGAK
								40.6	32.6		INVYYNEATGGK

Appendix 3

LC/MS/MS Analysis of 2D gel spots: Finding biomarkers for psychosis in plasma.

Spot No.	Protein I.D.	Species	Accession No.	MW (Da)	pI	No. Peptides Matched	Percentage Coverage	Sequence Matched
60	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin)	Human	P68871	15988	6.75	1	6%	LLVVYPWTQR
	Peroxiredoxin-1 (EC 1.11.1.15) (Thioredoxin peroxidase 2)	Human	Q06830	22096	8.27	1	5%	QITVNDLPVGR
61	Complement factor I precursor (EC 3.4.21.45) (C3B/C4B inactivator)	Human	P05156	65677	7.72	2	3%	VFSLQWGEVK AQLGDLPWQVAIK
	Clusterin precursor (Complement-associated protein SP-40,40)	Human	P10909	52461	5.89	1	3%	LFDSDPITVTVPVEVSR
64	No Identification Assigned							
67	Protein S100-A8 (S100 calcium-binding protein A8) (Calgranulin-A)	Human	P05109	10828	6.51	1	11%	LLETECPQYIR
72	Serum albumin precursor	Human	P02768	69321	5.92	26	42%	AEFAEVSK
	Gelsolin precursor (Actin-depolymerizing factor) (ADF) (Brevin) (AGEL)	Human	P06396	85644	5.9	2	5%	EVQGFESATFLGYFK QTQVSVLPEGGETPLFK
92	Ig kappa chain C region	Human	P01834	11602	5.58	5	67%	DSTYSLSSTLTLSK SGTASVVCLLNNFYPR VYACEVTHQGLSSPVTK TVAAPSVFIFPPSDEQLK VDNALQSGNSQESVTEQDSK
	Ig kappa chain V-II region TEW	Human	P01617	12308	5.69	3	38%	FSGSGSGTDFTLK ASGVPDRFSGSGSGTDFTLK DIVMTQSPLSLPVTPGEPASISCR
94	Ig kappa chain C region	Human	P01834	11602	5.58	2	32%	SGTASVVCLLNNFYPR TVAAPSVFIFPPSDEQLK
	Ig lambda chain C regions	Human	P01842	11230	6.92	2	37%	AAPSVTLFPPSSEELQANK ATLVCLISDFYPGAVTVAWK
1286	Afamin precursor (Alpha-albumin) (Alpha-Alb)	Human	P43652	69024	5.64	8	15%	FTFEYSR AIPVTQYLK FTDSENVCQER AESPEVCFNEESPK IAPQLSTEELVSLGEK SDVGFLPPFPTLDPEEK AESPEVCFNEESPKIGN SCCEEQNKVNCLQTR
1847	Complement C1r subcomponent precursor (EC 3.4.21.41)	Human	P00736	80122	5.89	17	32%	GYGFYTK
	Complement C4-A precursor (Acidic complement C4)	Human	P0C0L4	192650	6.65	10	9%	QGSFQGGFR